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Ontario. MOE.
TECHNOLOGY TRANSFER CONFERENCE #4. NOV. 29 & 30, 1983.
PART 1 - GENERAL RESEARCH. 1983.

ISBN - 0 -7743-8797-1

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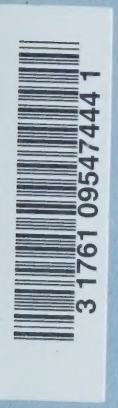
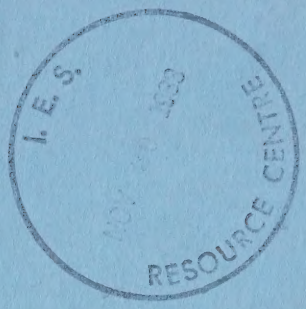
PART 1





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TECHNOLOGY TRANSFER CONFERENCE No.4

PART 1

GENERAL RESEARCH

CONSTELLATION HOTEL , TORONTO

NOVEMBER 29&30 , 1983

SPONSORED BY

THE RESEARCH ADVISORY COMMITTEE

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November 29 and 30, 1983

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Acknowledgements

The Ministry of the Environment's Research Advisory Committee would like to acknowledge the cooperation and efforts of the authors and Ministry staff who contributed to the organization of the Conference.

The financial support of the Provincial Lottery Trust Fund for environmental health-related research in Ontario has largely contributed to the success of this program including its technology transfer.

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PART 1

GENERAL RESEARCH

The Feasibility of the Sterile Male Approach To Control The Onion Maggot

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INTRODUCTION

The sterile insect release method (SIRM) has provided effective control of a variety of severe pests, eg., primary screw-worm fly (Cochliomyia hominovorax) and mediterranean fruit fly (Ceratitis capitata). The use of this method results in a significant reduction in environmental contamination with insecticides and a reduction in residues in produce. The SIRM, while costly, has been economically viable when used against insects which are difficult to control with the more classical techniques (physical, cultural and chemical control methods).

At present, the only effective means of controlling the onion maggot (Delia antiqua) in commercial fields is with the intensive use of insecticides. This species has shown a remarkable ability to develop resistance to insecticides. In order to compensate for this resistance, growers increase the application rate until the maximum rate is used or until phytotoxicity is evident. Increasing application rates only delays the time when a new insecticide must be found to replace the old. In Ontario, the onion maggot has developed resistance to specific cyclodiene, carbamate and organophosphate insecticides with any one insecticide being effective for only a few years. This "pesticide treadmill" has been ongoing for more than 20 years with the onion maggot. The treadmill will abruptly end when no new effective insecticides are available. This point is being rapidly approached as few new insecticides are being registered, especially on minor crops. As a result, an effective alternative means of control must be available.

This project was designed to determine if the SIRM was biologically possible and economically viable when used for the control of the onion maggot. During 1981 and 1982, the project focused on the development of: a) mass rearing techniques; b) release methods; and c) field evaluation techniques.

In 1983, these techniques were used to mass rear onion maggots for a release in July. This report summarizes the work of the past three years.

MASS REARING TECHNIQUES

Adult Flies

Two types of cages were used for the maintenance of adult flies. Twenty cages (48 x 48 x 59 cm) were constructed of plexiglass with an access port on one side and an opening to allow air movement on the opposite side. The access port was closed with a cotton sleeve and the air opening covered with nylon screen. The cages were designed to make maximum use of the environment chamber in Graham Hall. These cages were relatively large, awkward and expensive to build. Therefore, the 214 cages used in the environment chamber in the Biological Control Laboratory were constructed with 1.2 x 1.2 cm spruce and nylon screen. Each (44 x 32 x 49 cm) cage was accessed through a 12 x 12 cm entry port, closed with a cotton sleeve. The sleeve and entry port of each type of cage could be removed to allow free access for initiation or cleaning.

Each cage contained two watering devices. Each device was made of a clay pot (9 cm diam.) with a rubber stopper in the drain hole. The pot was filled with distilled water, and the top covered with a plastic petrie dish. The dish was secured to the pot with an elastic band and the device inverted. The water percolated through the clay pot from which the adult flies could obtain water. Two devices in each cage provided water for ca. two weeks. Solid protein (Table 1) was provided in petrie dishes as required. The best diet was identified as described in Appendix 1. A carbohydrate souce was provided as required by applying a mixture of honey, sucrose and yeast hydrolysate (Table 1) to the top of the cage. The yeast hydrolysate was added to thicken the mixture and prevent its dripping into the cage.

Two plastic containers, each containing 3,250 pupae mixed with moist vermiculite, were placed into each cage. The moist vermiculite was added to

prevent desiccation of the pupae. The containers with pupae were placed into the cages one day prior to the commencement of emergence. Eighty percent of the pupae emerged within four days of the start of emergence. Pupae reared for the colony had a mean emergence rate of 62 $\pm$ 10%. With this emergence success, ca. 4,500 adult flies were present in each cage after completion of emergence. This provided an absolute density of one fly/13.8 cm^3 in the plexiglass cages and one fly/19.4 cm^3 in the screen cages. These densities were used as they are in the optimum range for the space requirements for the onion maggot.

One week after the cage had been established, four oviposition dishes were placed into the cages with one in each corner. The adults frequent the corners of the cage and greater oviposition occurred if the dishes were placed in these areas. Each dish contained half an onion, with the cut-face partially buried in moist sand. This was found to be the most practical oviposition attractant as outlined in Appendix 2. The oviposition dishes were sprayed with water each day to maintain a high moisture level. This increased oviposition activity and decreased mortality of the first-instar larvae. The dishes were left in the cages for 72 h. By the end of this period some hatching had occurred and the first-instar larvae had entered the onion.

The adult flies were maintained for seven oviposition periods (21 days). Following this time, the watering devices and food dishes were removed and cleaned. The dead and surviving flies were removed, the cages washed and prepared for the next colony.

To ensure constant egg production, the 234 cages were divided into three groups of 78 cages each. Each group was started 12 to 14 days after the previous group. This provided a one-week pre-oviposition period, a three-week oviposition period and one week of free time for cleaning. The average egg production using this system was 2.4×10^6 eggs/week.

Larval Rearing Techniques

The onions used to feed the larvae were cut in half on their vertical axis with an automatic onion slicer. Cutting the onion on its vertical axis exposed the area preferred for feeding to the first-instar larvae, i.e., the meristemmatic tissue. In addition, more onions could be placed in each box when they were sliced in half.

Larvae were reared in corrugated cardboard boxes (46 x 60 x 15 cm) lined with a polyethylene sheet. A layer of moist vermiculite (2 cm deep) was placed over the liner. The onion halves were placed into the boxes with their roots down so that when the first-instar larvae migrated downward after hatching, they encountered an area of meristemmatic tissue. An average of 8.9 kg of onions were placed in each box.

To establish the mean oviposition rate per dish on a given day, oviposition dishes were randomly selected, removed from the cages and the eggs removed by flotation. These eggs were washed into a calibrated cylinder and the average number of eggs per dish was measured. The eggs and first-instar larvae in the oviposition dishes were washed with the sand and onion onto the top of the onion-filled box. Approximately 20,000 eggs were placed in each box as determined from the measured subsamples. A layer of moist vermiculite was spread over the eggs and onions so that the onions were completely covered. Seven L of vermiculite was used in each box. The box was placed onto rearing carts with 18 boxes/cart, and the cart moved into an incubator maintained at 23°C, photoperiod 8:16 L/D. The boxes were left in the incubator for 24 h during which time the eggs hatched and the first-instar larvae had the opportunity to migrate to the meristemmatic region of the onions. The box was left at 23°C if it was to produce nondiapause pupae. The nondiapause pupae could be used in the colony or released if insufficient time was available to terminate diapause. If the box was to produce diapause pupae, it was moved to an incubator maintained

at 11°C, photoperiod 0:24 L/D. Larvae were reared under these conditions, as larval survival was much improved, i.e., $\bar{x}$ = 47% at 11°C vs. $\bar{x}$ = 28% at 23°C, and diapause pupae could be stored more successfully.

When raised under diapause conditions, the larvae required re-feeding approximately 40 days after hatching. However, as the number of eggs placed into each box was only approximate, the rate at which the food was used varied significantly. To identify when each box was to be re-fed, onion halves were placed on the top of the vermiculite 35 days after hatching. These onions were checked each day and when larvae were found in these onions, the boxes were re-fed. Re-feeding required 7 kg onion halves and 4 L of dry vermiculite. The vermiculite was added to reduce the amount of free water in the box. This reduced mortality due to the production of anoxic conditions and reduced drowning. When re-feeding, the new onions were placed below those that had decomposed. A second re-feeding was required for larvae reared under diapause conditions approximately 15 days later. The same procedure was followed as that of the first re-feeding, however 3 kg of onion halves were added and 1 to 3 L of vermiculite used to help dry the boxes.

Larvae reared under nondiapause conditions were re-fed once, 10 days after hatching. The same procedure was used as described above for the first re-feed of the diapause group.

Pupal Harvest Technique

Sixty days after the initiation of diapause boxes or 21 days after initiation of nondiapause boxes, the pupae were harvested. The entire contents of the boxes were dumped into a 0.8 m diameter drum constructed of 2 x 2 cm steel mesh. The contents of three boxes could be accommodated in the drum at one time. After loading, the steel mesh door was closed, secured, and the drum rotated on a horizontal axis. An electric motor was used to drive the drum at approximately 30 rpm for one minute. As the vermiculite and decomposed onions tumbled in the

drum, the decomposed onions were broken into relatively small pieces. This allowed all of the pupae, vermiculite and small pieces of onion to fall through the mesh into a bin below the drum. As the drum was rotated, dry vermiculite was added so as to reduce the moisture content of the mixture.

The contents of the bin was shovelled onto the loading bins of two identical pupal separators. The separators consisted of a series of three horizontal screens aligned directly below each other. Each (54 x 180 cm) screen was constructed from a wood frame and steel mesh. The mesh sizes for the top, middle, and bottom screens were 8.0, 6.0 and 4.0 mm, respectively. The screens were held in a metal frame which provided support yet allowed the screens to move on a horizontal axis. The frame was attached to a camshaft driven by an electric motor which provided a horizontal shaking motion to the metal frame and screens. Each screen was sloped so that it had a drop of 7 cm over its length. Debris which could not pass through a screen was shaken down to its lower end and into collecting bins.

The vermiculite/pupae/onion debris mixture was gradually fed onto the top screen from the loading bin. The top screen stopped all debris larger than the pupae. The debris was collected, mixed with dry vermiculite and passed through the separator again to ensure the removal of all pupae. The pupae and vermiculite passed through the first screen and onto the second and third screens. These two screens retained all of the pupae but allowed the vermiculite to pass through. The vermiculite collected under the separator and was removed when necessary. When new larval rearing boxes were needed, the used vermiculite was sterilized and reused.

Flotation was used to remove organic debris and small lumps of vermiculite from the pupae. The pupae and debris were placed into a 50 x 50 x 30 cm metal tub which contained water to a depth of approximately 20 cm. The pupae were allowed to float to the surface and the debris settled to the bottom. The

settled material was stirred to release any pupae from the debris. The pupae were removed from the surface of the water with a sieve, dried and, if necessary, cleaned with a stream of air. The pupae and debris were dropped through a stream of air which blew debris of lighter or heavier density away from the pupae. The extraction efficiency of the major stages of the pupal harvest is shown in Table 2.

Pupal Storage

The above extraction procedure produced clean, dry pupae with very little debris. If significant debris was present during storage, fungal growth could occur and reduce pupal vitality. The pupae were stored in one-L plastic containers sealed with a plastic lid with 15,000 pupae/container. Both diapause and nondiapause pupae were held at 4°C until needed, nondiapause pupae for the colony or release, diapause pupae for release. The diapause pupae had to be stored a minimum of 60 days to fulfill the requirements of diapause development.

Nondiapause pupae were removed from storage 11 days prior to the initiation of new adult cages. The pupae were removed from their storage containers and split into four groups of approximately 3,250 pupae. The pupae were mixed with moist vermiculite, placed into 0.5-L containers, and held at 23°C. One day prior to the commencement of emergence, the new cages were initiated and the containers with pupae were placed into the cages.

STERILIZATION AND RELEASE METHODS

In 1982 and 1983, diapause pupae were removed from storage and placed at 23°C for 10 days. The containers containing pupae were placed into a Gamma Cell and exposed to 3 krad of γ radiation. A 60-second exposure was required with two containers irradiated at one time. This timing and rate of irradiation was identified as the optimum, as described in Appendix 3. Both males and

females were sterilized, however vitality was not affected.

In 1982, the irradiated pupae were mixed with moist vermiculite containing a red dye, placed into cages and allowed to emerge. The dye adhered to the ptilinum which was retracted shortly after emergence. In this manner all released flies were marked and could be identified after recapture. The adult flies were provided food and water as previously described. Thus the flies had the opportunity to feed and obtain moisture prior to the release, thereby increasing their ability to survive in the field.

Three days after the completion of emergence the cages were placed at $4^{\circ}C$, the flies removed from the cages and placed into one-L plastic containers. Each container held approximately 7,500 adult flies. Each container had two openings covered with nylon screen to allow for air movement. The containers were placed into coolers and maintained at $4^{\circ}C$ until weather conditions were appropriate for the release; i.e., wind spread < 10 km/h and temperature < $30^{\circ}C$. The flies did show significant mortality after 3 days of storage. When conditions were appropriate, the coolers containing an ice-pack and the pupae were transported to the Guelph airport. The flies were maintained at low temperatures to minimize their movement and respiration rate. They were loaded into the rear passenger compartment of a Cessna aircraft. Transportation to the airport required 20 min. and air-time to the release site required 30 min.

The flies were released over a 60-ha field from a height of approximately 50 m. The passenger removed the lid from the containers and poured the flies out of the passenger window. The aircraft was flown with 7° flaps so that the flies would not strike the plane after release. One million flies could be held and released on each flight. As this was a preliminary trial to test methodology, only one release of one million flies was made in 1982. This release was timed so as to correspond with emergence of the first portion

of the second generation, i.e., 10 July in 1982.

In 1983, the irradiated pupae were mixed with moist sand and placed into 25 x 32 x 10 cm plastic tray. All of the flies released on 12 July 1983 were reared under diapause conditions and were marked with pink dye. Flies released on 21 July 1983 were marked with either green (nondiapause flies) or blue (diapause flies). The dyes were mixed into 2 cm of sand which was placed on top of the sand and pupae in the tray. As in 1982, when the flies emerged through the dye, it adhered to the ptilinum and allowed identification of each group of flies when they were recaptured. The plastic tray containing the pupae, sand and dye were placed on shelves in a picnic table enclosure made of nylon screen. This structure was used as a cage for the flies and was located in a controlled environment chamber maintained at 23°C. After emergence the adult flies were provided access of food (Table 1) for at least three days prior to each release.

On the date of release, the temperature in the environment chamber was lowered to 4°C, minimizing the activity of the adult flies. All of the flies were removed from the cage by aspiration and placed into one-L plastic containers (the same as used in 1982). The containers were placed into 110-L plastic garbage pails along with ca. 9 kg of ice. The pails containing the adult flies were transported to the Keswick marsh in an airconditioned vehicle. The 3.0×10^6 flies in each release were distributed evenly throughout the release area between 19:00 h and 21:00 h. Five onion fields with a total area of 100 ha were used as the release area. Each of the five fields was divided into quadrants and an appropriate number of flies released in each quadrant so as to provide an even distribution of flies over the 100 ha. Previous trapping of adults had shown that the populations of onion maggots were similar in all five fields. The first release was timed by day-degree accumulations to coincide with the start of the second generation while the second release

on 21 July was to augment and extend the effect of the first release.

FIELD EVALUATION

Fertility

In 1983, live flies were captured with interception traps two weeks before and two weeks following the release. In 1983, live flies were captured from three weeks before the first release to five weeks after the second release. Each trap was made of a metal frame 1.4 m high, 1.0 m wide and 0.3 m deep. The top and three sides were covered with nylon screen leaving one long side open. The adult flies would fly into the open side, encounter the screen and fly up to the top of the trap. Two glass jars were located at the top corners of the trap. Each jar contained a funnel which allowed the flies to enter, but not leave the jar. The traps were located at four locations, one set of traps on each of the four borders of the field. Two traps were placed at each location, one trap facing towards the crop and the other towards the hedgerow. In 1982, interception traps were placed around the treated field and around a 20-ha control field located 6 km away from the treated field. The same control field was used in 1983 and the treated (release) field was chosen at random from the 5 release fields. In both 1982 and 1983, the control field received insecticide treatments as deemed necessary by the grower, i.e., three treatments with diazinon during the second generation. The interception traps were checked 5 days a week. When checked, the bottles on the top of the traps were removed, sealed and replaced with empty jars. The sealed jars containing flies were returned to the field laboratory where they were anaesthetized with CO_2. The male flies were identified, their ptilinum everted and examined for dye. This process resulted in the death of the fly. The females were placed in a 910 cm^3 cage which was provided with food, moisture and an oviposition dish. Every two days thereafter the oviposition dish

was removed and replaced with a new dish. The eggs were removed from the oviposition dish by flotation. They were placed into a petri dish (5 cm diam.) which was sealed and held at 23°C for three days. The proportion of the eggs hatching was determined by counting the total number of eggs and the number hatched three days after oviposition. After fertility was checked, the female flies were killed and the ptilinum everted and checked for dye. From previous laboratory studies, it was ascertained that sterilized released flies did not produce eggs. Therefore, all eggs layed were from the wild population.

Population Monitoring

Populations of adult flies in the treated and control fields were monitored using sticky traps. These traps were placed in the onion fields as early as possible after seeding and checked throughout the summer until the crop was windrowed. Each trap consisted of three wooden stakes (2.5 x 5.0 x 121 cm) placed in a row with 0.3 m between stakes. One-L milk cartons were painted yellow, coated with Tangletrap and placed into a notch on each stake. The cartons were kept just above the onion canopy to maintain trapping efficiency. The cartons were replaced at 10- to 15-day intervals or more frequently if they became covered with dust or large numbers of insects. The number of traps per field was dependent on field size. In 1982, the 60-ha treated field had 16 traps while the 20-ha field had 4 traps. In 1983, each field was trapped as follows: 40 ha - 8 traps, 2-20 ha fields - 8 traps, 15 ha field - 6 traps, 4 ha field - 4 traps. Each trap was randomly positioned along the sides of the field. The traps were examined two or three times each week throughout the growing season. Traps were checked at approximately the same time each day to prevent errors in fly counts caused by periodicity of flight activity. When the traps were checked, the flies were removed with a scraper and placed on a piece

of bristol board. The flies were returned to the lab and their ptilinum checked for dye. In this manner the total number of flies caught and the proportion marked was determined.

The proportion of marked to unmarked flies caught in the interception and sticky traps provided the data to calculate a Lincoln Index.

Crop Damage

Crop damage was assessed in the treated and control fields using plots containing 100 onions. Each plot consisted of four double rows with 25 plants per row. Wooden stakes (25 cm x 5 cm) were placed in each row to denote the 100 onions in the plot. A red flag attached to a 1-m flexible rod was placed at each plot so that they could be found after the crop had grown. Ten plots were randomly placed in the field. The damage plots were examined after the first and second generations to determine the number of plants damaged by onion maggots. After the second generation the damage plots were removed. Damage to the crop at harvest, i.e., late second- and early third-generation damage was assessed when the onions were in windrows immediately prior to harvest. Ten samples of 100 onions each were examined for damage. The samples were taken near where the damage plots were located. Damage in the control and treated fields was compared after each generation and found not to be significantly different ($P \geq 0.05$).

The above studies (fertility, population size and crop damage) were found to be adequate to determine the efficacy of the sterile insect release method in comparison to normal grower practices.

SUMMARY

The techniques have been developed and tested for the mass rearing, sterilization, release and field evaluation of the sterile insect release method for control of the onion maggot. These techniques were used in 1983

for a large release against the second generation of the onion maggot in the Keswick Marsh. Six million flies were released resulting in a reduced fertility of ca. 35% for 3.5 weeks in the release area. (A complete report of the data is not possible at this time as it has not been fully analyzed). These data indicate that the use of the SIRM technique is a possible alternate control technique for the onion maggot. However, due to the relatively high cost of this method it is unlikely that it can be used on a large scale.

Table 1. Diets used for the maintenance of adult onion maggots.

"Protein Diet"	
	50% yeast hydrolysate
	50% Brewer's yeast
"Honey Diet"	
	80% honey
	13% sucrose
	7% yeast hydrolysate

Table 2. Number of pupae not extracted from the larval rearing media.

	Pupae Not Extracted	Percent Not Extracted
Drum	1,109	2.2
Shaker	3,126	6.3
Total	4,235	8.8

(Total number extracted 50,000)

APPENDIX 1. Effects of Diet and Cage Location on Oviposition of Delia antiqua.

Object: To determine the effect of diet and cage location on viable egg production by Delia antiqua.

Materials & Methods: The cages were established as follows:

- 16 screen cages
- 4 oviposition sites/cage
- 2 watering devices/cage
- 7,500 diapause pupae/cage
- free access to honey at the top of each cage
- free access to the specified diet

Diets used:

1. Hydrolysate Diet
   - 50% yeast hydrolysate
   - 50% Brewer's yeast

2. London Diet
   - 50% Brewer's yeast
   - 33% yeast hydrolysate
   - 17% soya flour

3. Guelph Diet
   - 37.5% soya flour
   - 25.0% sucrose
   - 12.5% Brewer's yeast
   - 12.5% yeast hydrolysate
   - 12.5% soya protein

4. Ticheler - Dutch Diet
   - 45% milk powder
   - 45% sucrose
   - 5% Brewer's yeast
   - 5% soya peptone

- The number of dead flies was counted before the first and after each 2-day oviposition period so that the number of viable eggs/living female could be determined for each period.

- A 4 x 4 latin square design was used with four diets assigned to each row and column of cages. Each diet was represented once in each column and row.

- Oviposition dishes were placed into cages for 13 periods with two days per period. The number of eggs oviposited in each cage, each period, was determined volumetrically.

- Fifty eggs from each cage were placed into petri dishes and checked for hatching five days later.

- The hypothesis was listed as a three-factor ANOVA with 13 replicates.

Results: Diet did affect egg production as shown below:

Diet	Viable Eggs/Female/Period*
1. Hydrolysate	3.58^{a}
2. London	3.11^{a}
3. Guelph	2.53^{b}
4. Dutch	1.43^{c}

*n = 52; means followed by different letters are significantly different ($P \leq 0.05$).

-There was no significant effect ($P \leq 0.05$) of row or column location on egg production.

Conclusions: The London and Hydrolysate diets were the best.

- Cage location had no significant effect on egg production.

APPENDIX 2. The Effect of Oviposition Attractants on Egg Production by Delia antiqua.

Object: To determine if specific natural, synthetic chemicals or fresh onion halves would enhance egg production by Delia antiqua.

Materials & Methods: The cages were established as follows:

- 16 screen cages
- 2 watering devices/cage
- 7,500 diapause pupae/cage
- free access to the Hydrolysate diet and honey
- 4 oviposition sites/cage; one in each corner

- The oviposition site consisted of a 9 cm diam. x 25 mm deep plastic petri cish. Each dish contained 50 ml of washed #24 silica sand and an attractant. After the attractant was added, 1/2 of a 6 cm diam. onion was partially buried in the sand with the cut face down.

- The chemical treatments were:

  1. 2.5 ml of 10% propanethol in methanol.

  2. 2.5 ml of 1% propyl disulfide in methanol.

  3. Rotting onions, obtained from the larval rearing boxes, were homogenized, the resulting fluid filtered and 2.5 ml of this fluid applied to the sand for Treatment 3.

  4. The control, which received only methanol.

- All of the treatments were moistened with a total of 12.5 ml water.

- One of each of the four treatments was placed in each of the 16 cages. One treatment was placed in each corner. The dishes were removed every two days and replaced with an appropriate treatment. The orientation of the treatments in the cages was based on a latin square design which was rotated. This allowed any one treatment to occupy one corner only once.

- Eight periods were run for a total of 16 days. This provided data for two latin square designs.

- After the dishes were removed from the cages, the eggs were removed by flotation and counted.

- These data were analysed using a three-factor ANOVA as a vested design. Egg deposition/period was the unit of measure.

Results: A significant difference was evident among the oviposition attractants as shown below.

Attractant	Average Number of Eggs/Period*
1. propanethol	143.8^{a}
2. propyl disulfide	124.1^{a}
3. onion rot	48.3^{c}
4. control	96.6^{b}

*n = 128; means followed by different letters are significantly different ($P \leq 0.05$).

Conclusions: The first and second treatments were more attractive than the half onion alone or onion and onion rot. However, due to the severe smell of these attractants and the relatively small difference as compared to the control. The control treatment was chosen for use in the colony.

APPENDIX 3. Sterilization of Delia antiqua.

Object: To identify the optimum rate and timing of irradiation of pupae of Delia antiqua in order to produce sterile adults.

Materials & Methods: Approximately 10,000 pupae were removed from storage and divided into two groups of equal size; one group to be eradicated, the other for a control.

- The pupae destined for irradiation were divided into six groups of equal size.
- All pupae were mixed with moist vermiculite and maintained at room temperature in one-L plastic containers.
- Nine and 12 days after removal from storage, one container with pupae was irradiated at each of 3,4 & 5 krad of irradiation.
- As irradiated flies emerged, they were separated on the basis of time of irradiation in relation to emergence (i.e., 2, 4, 6 & 8 days prior to eclosion) and by sex.
- The flies were placed into 910 cm^3 cages for crosses with flies from the control group. These cages contained food, a water source and an oviposition site.
- The sex ratio in all crosses was maintained at 1:1 with the number of flies per cross varying from 2 to 25. This was dependent upon the number of flies available on a specific day for each cross.
- The oviposition sites were removed every second day, the eggs separated by flotation and visibly counted.
- Egg fertility was established by holding the eggs for 5 days and counting the number of eggs which hatched.
- The spermatheca of males were removed, placed into a "wet mount", squashed and the presence or absence of motile sperm noted.

Results: There were no significant differences among the crosses resulting from pupae of different age (i.e., the time when the pupae were irradiated did not affect the results).

Table 1. Results from the crosses of sterilized females and fertile control males.

Radiation Dose (krad)	No. of Females Tested	No. of Eggs Laid	No. of Eggs Hatching	Percent Viability	No. of Eggs/Female
3	70	1	0	0	0.01
4	117	0	0	0	0
5	90	24	1	4.2	0.3

Table 2. Results from the crosses of sterilized males and fertile (control) females.

Radiation Dose (krad)	No. of Females Tested	No. of Eggs Laid	No. of Eggs Hatching	Percent Viability	No. of Eggs/Female
3	92	1,077	0	0	11.7
4	102	1,407	4	0.3	13.8
5	72	1,218	2	0.2	16.9

- The above tables indicate no significant difference among the 3 radiation doses. All resulted in very low viable egg production.
- Egg production of sterilized females was much reduced in comparison to control of females.

Table 3. Reproductive ability of control and sterile crosses.

Cross	No.of Females	No. of Eggs Laid	No. of Eggs Hatched	Percent Viability	No. of Eggs/Female	Insemination Rate (%)
♀ (virgin)	75	323	0	0	4.3	-
♂ x ♀	104	2,121	1,058	49.9	20.4	51.9
♂ x ●	277	25	1	4.0	0.1	47.4
♂ x ♀	266	3,702	6	0.2	13.9	34.0

♀ control female ♂ control male
● sterile female ♂ sterile female

- Untreated virgin females will lay eggs.
- The "control" cross (fertile male x fertile female) produced 20.4 eggs/female with an insemination of 51.9%.
- Sterile females have a much reduced egg production of 0.1 eggs/female but a similar incidence of insemination.
- Control females inseminated by sterile males laid eggs at a rate pf 13.9 eggs/female but these had less than 1% hatch.
- The insemination rate of these females was similar to but lower than the control.
- All observations concerning competitive ability and longevity showed no noticeable difference between treated and control flies.

Conclusions: Any treatment of 3 krad or more of any of the pupae listed produced effective sterility of both male and female onion maggots. The vitality of the treated flies was not reduced.

DEVELOPMENT OF METHODS FOR THE DETECTION OF ROTAVIRUSES

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Rotavirus is the major etiological agent of infantile gastroenteritis, and is associated with up to 50% of the hospitalized cases of diarrheal illness in infants and young children. Between 1972-1979 there were 26 fatal rotavirus infections recorded at Toronto's Hospital for Sick Children. Because large quantities of virus are shed in the stool during infection, and because of the hardy nature of the virus, it is not surprising that rotavirus pollution of public water has become a matter of considerable concern. Sensitive virus detection methods are essential for monitoring the distribution of rotavirus in the environment. It has been the objective of this project to develop laboratory methods for the detection of rotavirus, and to design these methods so that they could be applicable to environmental samples.

In the initial phase of the project, simian rotavirus SA-11 was used as the virus model, and methods were investigated for detecting small quantities of rotavirus that had been isolated in cell cultures. Two new light microscope methods were developed: (1) the SPA method employing fixed Staphylococcus aureus cells as morphological markers; (2) a fluorescent antibody method (LAB) utilizing the high affinity of avidin for biotin. Both methods were compared with the more commonly used indirect immunofluorescence method (IIF).

The LAB method was found to offer the greatest sensitivity. It could detect, after 24 hours incubation, intracellular multiplication of an inoculum as low as 100 $TCID_{50}$, whereas the minimum inoculum size detectable by SPA was 125, and by IIF was 170. When approximately 20,000 $TCID_{50}$ of rotavirus was inoculated into cell cultures, its presence could be detected at 4 hours by LAB, 1 hour earlier than by the other two methods.

Current procedures for detecting viruses in the environment rely heavily on cell culture isolation. But by this approach there is danger of excluding several important viruses which do not grow readily in cell culture. Best known of these fastidious viruses are hepatitis A virus, Norwalk virus, certain strains of adenovirus, and human rotavirus. The next phase of this project should concern itself with the application of methods such as the LAB method, as well as immunoelectron microscopy and enzyme-linked immunosorbent assay (ELISA), for direct detection of viruses in environmental samples, thus bypassing the extremely useful but highly selective cell culture isolation step.

INTRODUCTION

The present study has been part of a long-term project to develop sensitive and reliable methods for detecting viruses that might pollute drinking and recreational water. The specific objective of the present study has been to develop a method for detecting rotaviruses.

Rotaviruses constitute one of the most serious causes of gastrointestinal infections among infants and young children. Besides causing a great deal of morbidity, rotaviruses may also cause fatal disease. Between 1972 and 1979, 24 fatal cases of rotavirus were documented at the Hospital for Sick Children (Middleton 1982).

Diarrhoea is the main clinical feature of rotavirus infections, and large quantities of virus are shed in the stools. Being non-enveloped, rotaviruses are relatively hardy viruses, consequently they can remain infectious for long periods of time, especially when adsorbed to particulate material.

Despite the fact that rotaviruses are responsible for such a large proportion of gastrointestinal infections, especially among infants and neonates, laboratory studies on these viruses have been slow in coming. Rotavirus is one of the "newer" viruses, and it was not discovered, by laboratory methods, until 1973 (Bishop et al., 1973; Flewett et al., 1973). This long delay in detecting a virus that causes such a high incidence of morbidity has been due to the fact that human rotavirus cannot be grown by the usual tissue culture methods employed routinely for other viral pathogens.

To date there has been only limited success in growing human rotaviruses in tissue culture, and this has required such methods as

preliminary passage of the virus in animals (Wyatt et al, 1976; 1980), and centrifugation of the inoculum onto susceptible cell monolayers (Banatvala et al., 1975; Bryden et al., 1977). Animal strains of rotavirus have been less fastidious, consequently studies on rotaviruses and rotavirus infections have dealt primarily with non-human strains, such as Nebraska calf diarrhoea virus (NCDV), and simian rotavirus SA-11. Both of these viruses grow well in cell culture. Furthermore - and of particular importance - certain viral antigens are common to all species of rotaviruses, consequently detection of human rotavirus antigens and antibiodies can be carried out using e.g. NCDV or SA-11 reference strains.

OBJECTIVES

The immediate objective of this study has been to develop a sensitive and reliable method for rotavirus detection that would be applicable to environmental samples. A variety of laboratory methods are currently in use for detecting rotavirus in clinical specimens (faeces), and these include:

A. Direct examination of the specimen for the presence of virus particles or viral antigens by -
   (i) electron microscopy (EM)
   (ii) enzyme-linked immunosorbent assay (ELISA)
   (iii) counterimmuno electrophoresis (CIOP)

B. Inoculation of the specimen into cell cultures in order to amplify the virus content (applicable only to rotavirus strains that can be grown in cell culture), then detection and identification of virus by -

(i) electron microscopy

(ii) ELISA

(iii) fluorescent antibody technique (immunofluorescence)

The approach to the immediate objective has been as follows:

STAGE 1. Using NCDV and SA-11 develop a method that will give improved detection sensitivity relative to existing standard methods.

STAGE 2. Apply this method to the detection of rotaviruses in environmental samples (rather than clinical specimens).

STAGE 3. Develop methods for the isolation, detection and growth of human rotavirus strains.

REPORT OF STAGE ONE OF ROTAVIRUS PROJECT

Materials and Methods

Viruses

NCDV rotavirus was obtained from Dr. F. Bishai, Central Laboratory of the Ontario Public Health Laboratories. It was maintained by passage in BSC-1 cell cultures. To prepare virus pools, TC flask cultures of BSC-1 cells were washed twice with phosphate buffered saline (PBS), and inoculated with a dilution of virus calculated to give a 3+ CPE within 24 hr. Virus was allowed to adsorb for 1 hr. at room temperature. Maintenance medium consisting of MEM + HEPES buffer + 5 µg trypsin/ml + 0.5 - 0.75% bovine serum albumen (BSA) was added, and flasks were incubated at 37°C. At 3+ CPE, the cells were frozen and thawed 3 times, centrifuged at 1100 rpm for 10 min, and the supernatant removed and used as the stock virus pool (stored at -70°C).

SA-11 virus was obtained from Dr. L. Spence, Toronto General Hospital, and was grown in MA-104 cell cultures. To prepare virus pools, TC flask cultures of MA-104 cells were washed 3 times with PBS and inoculated with 1-2 ml of SA-11 virus that had been treated for 20 min with 10 µg/ml of trypsin. Virus was allowed to adsorb for 1 hr at 37°C before the addition of a serum-free nutrient medium consisting of Eagle's minimum essential medium (MEM) prepared with HEPES buffer, supplemented with 5 µg/ml of trypsin and 0.75% bovine serum albumin (BSA). Cells were incubated until a 3+ CPE was observed. They were then taken through three cycles of freezing and thawing, the cell lysate was centrifuged at 1100 rpm for 10 min, and the supernatant was removed and used as the stock virus pool (stored at - 70°C).

Purified virus pools were prepared by cesium chloride isopyric centrifugation, according to the method of Petric et al. (1975).

Cell Cultures

MA-104 cells (established fetal rhesus monkey kidney cells) were obtained from Dr. P.J. Middleton, Hospital for Sick Children. Cultures were prepared in 75 or 150 cc plastic flasks in a growth medium consisting of supplemented Eagle's minimum essential medium (MEM) in HEPES buffer, plus 5% fetal calf serum, and gentamycin. Two days after trypsination the cells were changed to maintenance medium, which was the same as the propagating medium except for a reduction to 2% fetal calf serum.

Both stationary and roller tube coverslip cultures of MA-104 cells were used. Lab-Tek tissue culture chambers (Miles Laboratories, Rexdale, Ontario) were incubated in a moist atmosphere at 37°C, in the presence of 5% CO_2. Glass coverslips, approximately 3 x 15 mm in size, were inserted in rubber-stoppered tissue culture tubes. Coverslip cultures were rotated in a roller drum at 1/3 rpm at 37°C for 2 days, a procedure that has been shown to enhance rotavirus infectivity (Sato et al., 1981, 1982). Cultures were then ready for virus inoculation.

BSC-1 cells (established African green monkey kidney cells) were obtained from Mr. Goff Jenkins, Ontario Ministry of the Environment. They were grown in MEM + HEPES buffer, supplemented with 5-10% calf serum, 10% tryptose phosphate broth, and gentamycin. Maintenance medium contained 2-5% calf serum.

Electron Microscopy

Samples to be examined by electron microscopy were processed by the agar diffusion negative staining technique (Anderson and Doane, 1972)

as follows: a 25 µl drop was placed on a plastic-coated EM specimen grid resting on 1% agar. When the drop had dried (45-60 min) the grid was removed, exposed briefly to 2% phosphotungstic acid pH 7 (negative stain), air-dried, and examined in a Philips EM 300 transmission electron microscope.

Indirect Immunofluorescence (IIF) Method

Coverslip cultures were washed with PBS, and inoculated with 0.1 ml of SA-11 rotavirus stock. Virus was allowed to adsorb at 4°C for 1-2 hr. At a predetermined time the fluid was withdrawn from the culture tube, the cells were washed three times with PBS, and fixed at -20°C for 10 min in absolute ethanol. The pH was adjusted by rinsing in PBS. The coverslip was then removed from the tube and placed, face up, on moistened filter paper in a petri dish. Rabbit anti-NCDV hyperimmune antiserum (obtained from Dr. L. Spence) was layered on top of the culture (25 µl of antiserum) and incubated at 37°C for 40 min. The culture was washed at least three times in PBS, then layered with 0.1 ml of goat anti-rabbit immunoglobulin antiserum that had been conjugated to fluorescein isothiocyanate (FITC) (Antibodies Inc., Calif.). After 40 min at 37°C the coverslip was rinsed in PBS, dried in air, mounted on a glass slide in fluorescent antibody medium pH 7.2 (Difco Laboratories, Detroit, Mich.), and sealed with nail polish. The mounted preparation was examined in a Zeiss incident-light fluorescence microscope equipped with a filter combination 48 77 09 + in the blue - ultraviolet excitation range 450-490 nm.

Experimental Procedures and Results

A. Development of a Protein A Method for Rotavirus Detection

The IIF immunoassay is a standard method that is used universally for detection of viral antigens (Gardner & McQuillin 1980). It is generally employed in the indirect form, which requires a primary anti-viral antiserum and a secondary anti-species antiserum that has been conjugated to FITC. It was our intention to replace the secondary reagent with a preparation of Staph. aureus cells, which have a unique protein (A) on the bacterial cell wall that has a strong affinity for immunoglobulins of many animal species (Mallinson et al., 1976). Protein A in purified form has been used extensively in immunoassays; however, in this study we elected to use the intact (fixed) bacterial cell, employing the bacterium itself as a morphological marker or label at the light microscope level (Goding 1978; Lancz & Specter 1982; Morgenson & Dishon; Surolia et al., 1982).

A variety of experiments were carried out, to investigate the suitability of the SPA method for rotavirus detection, and to compare the Staph. aureus Protein A (SPA) method with the indirect immunofluorescence test.

1. Preparation of Staphylococcus aureus Protein A. The Cowan strain of Staph. aureus ATCC 25904 was obtained from Dr. S. Vas, Toronto Western Hospital. Four colonies grown on blood agar plates were transferred to 5 ml of Todd-Hewitt broth and grown overnight at 37°C. This was then added to 100 ml of Todd-Hewitt broth and incubated for 18 hr at 37°C. The resultant dense culture was dispensed into centrifugation tubes and spun at 3000 rpm for 20 min. The supernatant was removed and a whitish

pellet with a greyish ring remained. The pellet was resuspended in approximately 20 ml of PBS, and the last procedure was repeated twice. The final pellet was resuspended in 2% formaldehyde in PBS and left for 3 hr at room temperature. This suspension was spun down and the pellet washed twice in PBS then resuspended in 5 ml of PBS and placed in a water bath at 80°C for 10 min. After centrifugation the pellet was resuspended in 10% w/v of PBS containing 0.1% sodium azide. A sterility check was performed before storing the preparation at 4°C.

The concentration of bacterial cells was determined by counting the cells in a microchamber, using light microscopy.

2. Basic procedure for detecting rotavirus in cell culture, using the SPA method. Virus inoculation and subsequent treatment of coverslip cultures was performed essentially as described above for IIF. After exposure to antiviral antiserum, cultures were rinsed in PBS and exposed to an appropriate dilution of SPA at 37°C for $\geq$ 30 min. Cultures were then rinsed in PBS, stained for 5 min with the fluorochrome dye acridine orange (AO), according to the method of Goldner et al., 1983, rinsed in citrate - phosphate buffer and mounted.

Results. Control and uninfected cultures of MA-104 cells treated with SPA and stained with acridine orange exhibited orange cytoplasm, yellow-green nuclei and orange nucleoli. Infected cultures treated with SPA and stained with AO contained foci of cells covered with orange-staining bacterial cells. The cocci appeared to be confined entirely to areas over the cytoplasm, with none being seen on the surface over nuclei.

3. Establishing the specific activity of the SPA method. The specific activity of the SPA detection method was demonstrated by a series of controls. Results are given in Table 1. It can be seen that a positive result was obtained only in the presence of a combination of virus, viral antibody, and SPA.

4. Establishing the specific activity of the IIF method. The specific activity of the IIF method was also investigated, in order to assess more fully the advantages and disadvantages of the two rotavirus detection methods. In the IIF method, the presence of antigen is indicated by the presence of fluorescence; there should be little or no "background" (non-specific) fluorescence. As shown in Table 2, there was a strong background fluorescence in cell control and infected cultures stained with FITC.

5. Determination of optimum concentrations of antiserum and SPA. Chessboard titrations of varying concentrations of anti-NCDV antiserum and SPA were tested, in order to determine the optimal concentrations required to give maximum labelling of infected MA-104 cells. As shown in Table 3, when SPA cells were used at a concentration of 3×10^{10}, maximum labelling was achieved with antiserum dilutions $\leq 1/80$; if antiviral antiserum was diluted byeond 1/80, the distribution of label decreased. SPA cells at a concentration of 1.5×10^{9} also produced maximum labelling, provided the antiviral antiserum was not diluted beyond 1/50. When SPA cell concentrations $< 1.5 \times 10^{9}$ were used, sub-optimal labelling was obtained.

At SPA cell concentrations $>1 \times 10^{10}$, non-specific clumping of bacterial cells occurred; this was not a problem when SPA cell concentrations

were < 2×10^9. Consequently, the optimum SPA concentration to use was estimated to be approximately 1.5×10^9 cells/ml.

6. Determination of optimum incubation times for antiserum and SPA. It was found that, to obtain maximum SPA labelling, it was necessary to incubate infected cells in the presence of antiviral antiserum for ≥ 40 min (Table 4). The SPA incubation period required to produce a positive reading was ≥ 20 min, with maximum labelling occurring when both antiserum and SPA were incubated for 40 min or more. On the basis of these experiments it was estimated that antiserum and SPA incubation periods should each be 60 min.

7. Comparison of SPA sensitivity versus other rotavirus detection methods. The SPA detection method was compared with several other methods to determine their relative sensitivity in detecting the presence of rotavirus. Two separate experiments were performed. The first involved the inoculation of known amounts of SA-11 virus into MA-104 cell cultures; after 24 hr the cultures were examined for rotavirus by each of three light microscope methods - cytopathic effect (CPE), IIF and SPA. It can be seen from Table 5 that the detection end point for all three methods was 10^{-6}. However, of greater importance was the observation that when an inoculum dilution ≤ 10^{-3} was used, both the IIF and SPA methods were more sensitive than CPE in detecting the presence of virus, with SPA being slightly more sensitive than IIF. When one calculates, by the Kärber method, the virus titre obtained by each method, there is almost a 2 log improvement in sensitivity in favour of the immunoassays.

In the second experiment carried out to compare the sensitivities of the different methods, a determination was made of the minimum time required, post-inoculation, for virus to be detected. As shown in Fig. 1, virus was first detected at 6 hr by both IIF and SPA. In contrast, a clear CPE was not evident until approximately 15 hr.

B. Enhancement of the Fluorescein Signal in the IIF Method

Technical problems with the IIF method were encountered in the earlier stages of this project, including extensive non-specific background fluorescence, and fading of the fluorescence during the long photographic exposure times required for the weak signal. Thus, although the IIF method proved to be almost as sensitive as SPA for rotavirus detection, the FITC signal was much more difficult to record, both visually and photographically. The purpose of this phase of the project was to attempt to overcome some of these problems by increasing the signal to noise ratio and amplifying and stabilizing the FITC signal.

It was found that background fluorescence could be reduced by incorporating 1% bovine serum albumen (BSA) in the PBS washes employed immediately pre-and post-fixation, prior to the addition of antiviral antiserum. The background fluorescence could be reduced even further by counter staining with 0.0001% Congo red in PBS, for 5 min at 22^{o}C, prior to mounting (Gardner and McQuillin, 1980).

The problem of fading of fluorescence during photographic exposure was eliminated by using the chemical antibleach para-phenylanediamine (Johnson et al., 1981, 1982) incorporated in a mounting medium of glycerol and Moviol (Hoechst Canada Inc., Montreal, P.Q.). It was also

found that by using Kodak Ektachrome daylight film of ASA 200 or ASA 400, rather than tungsten film of ASA 160, the exposure time for IIF could be reduced. In combination, these factors improved considerably the usefulness of the IIF method.

C. Development of a Labelled Avidin-Biotin (LAB) Immunoassay

The LAB immunoassay has been introduced only recently into diagnostic virology, and has the potential of being one of the most sensitive of those methods used to detect antigens. Avidin is a 68,000 molecular weight glycoprotein from egg white which has an extremely high affinity for biotin (vitamin H or coenzyme R) (Heitzmann & Richards, 1974; Green 1975; Bayer & Wilchek 1980), the avidin-biotin association constant being 10^{-15} M (compared to 10^{-5} to 10^{-9} M for antigen-antibody associations). Biotin can be covalently coupled to free amino groups on a protein such as IgG, and avidin can be conjugated to light microscope labels such as horseradish peroxidase (Guesdon et al., 1979) or FITC (Berman & Basch, 1980). Label amplification occurs as a result of the presence of four biotin binding sites on the avidin molecule (Green 1975). The 3-stage LAB assay used in our study (Figure 2) involved (1) an initial antigen-antibody interaction, (2) the addition of an anti-species antibody conjugated to biotin, and (3) the addition of an avidin-FITC conjugate.

LAB procedure. Coverslip cultures were washed twice in 1% BSA in PBS for a total of 10 min. Cells were fixed for 5 min in acetone, rinsed in 1% BSA in PBS, and coverslips were transferred to petri dishes containing moistened filter paper. A volume of 25 µl of antiviral antiserum (rabbit) was placed on each coverslip, which was then incubated at 37°C for 40 min.

After a brief PBS wash, cultures were exposed to 25 µl of antispecies antibody (goat anti-rabbit) conjugated to biotin (Cappel Laboratories, West Chester, P.A.) for 30 min at 37°C, washed again in PBS, incubated at 37°C for 30 min in the presence of avidin-FITC conjugate (Miles Laboratories, Elkhart, Ind.), washed 3 x in PBS, counterstained with 0.001% Congo red for 5 min, rinsed in PBS, air-dried thoroughly, and finally mounted in Moviol-glycerol-antibleach medium. Appropriate controls, comparable to those used for IIF and SPA, were used to ensure the specific activity of the LAB assay. Microscopy and photomicrography procedures were as described above for IIF.

Results. MA-104 cells infected for 18 hr with SA-11 virus and processed by the LAB method contained cells with discrete, bright areas of fluorescence scattered throughout the cytoplasm. In control and in infected cultures exposed to LAB, there was a uniform distribution of non-specific, faint background fluorescence (Table 6). However, its appearance was in marked contrast to the discrete fluorescing areas of viral antigen, and the two could easily be differentiated. Results of a chessboard titration carried out to determine the optimum concentrations of the conjugates that would give the best signal: noise ratio are given in Table 7. From these results it was determined that the optimum dilution of biotin-conjugated antiserum was 1/1500 and that of avidin-FITC was 1/50.

D. Comparison of IIf, SPA and LAB Methods for Rotavirus Detection

At this final stage of the first portion of the project, the three immunoassays - IIF, SPA and LAB - had been raised to maximum efficiency for detecting rotavirus antigen in cell culture. It was now necessary to determine which of the three methods would be most suitable as a

routine detection method. The following aspects of each method were investigated: (1) the earliest detection of antigen in infected cells; (2) the minimum amount of virus inoculum that could be detected 24 hours post inoculation (p.i.); (3) the minimum amount of antiviral antiserum required for a positive reaction; (4) the quality of the fluorescent signal, as recorded visually and photographically; (5) the cost and level of difficulty of setting up the test.

Results. When MA-104 cell cultures were inoculated with a multiplicity of infection of 1:25 (2×10^4 $TCID_{50}$/0.1 ml), viral antigen was first detected 4 hr p.i., by the LAB method only. One hour later, at 5 hr p.i., antigen was detected by all three methods (Table 8). The minimum inoculum that could be detected 24 hr p.i. was approximately 100 $TCID_{50}$, and this was detected only by the LAB method. The minimum inoculum required for IIF was approximately 125 $TCID_{50}$, and was approximately 175 $TCID_{50}$ for SPA. The LAB method required the smallest amount of antiviral antiserum, and could be used at almost twice the dilution required for SPA, and 1 1/2 times less than that needed for IIF.

DISCUSSION AND RECOMMENDATIONS

In a recent study group report published by the International Association for Water Polution Research (1983), the following recommendations were made:

"Virological research concerned with the optomization of monitoring systems and quality limits should include the improvement of techniques for the recovery and detection of small numbers of viruses in large volumes of water. Special attention should obviously be given to gastroenteritis viruses such as Norwalk- and rotaviruses, and the hepatitis A virus. These techniques are necessary to study the incidence and behaviour of viruses in water, and to establish relations between viruses and indicators, which are essential to develop reliable surveillance systems and quality limits which may not include virus tests." It has been the objective of the study reported here to develop such methodology for rotavirus detection. Because of our rather extensive experience with the simian rotavirus SA-11 (Quan 1982; Quan and Doane, 1983) we have used this virus in MA-104 cells as our model system throughout the first phase of the study. The standard cell culture detection procedures used as reference methods against which we compared our methodology were (a) cytopathic effect (CPE) and (b) indirect immunofluorescence (IIF).

IIF is undoubtedly one of the most important immunoassays used in diagnostic virology (Gardner and McQuillin 1980). However, IIF requires a great deal of "fine-tuning" to eliminate problems such as non-specific or background fluorescence in order to ensure reliable specificity coincident with high sensitivity. We were able to reduce background

fluorescence by the introduction of two steps: (1) the use of a 1% bovine serum albumin - PBS wash prior to the addition of FITC label, and (2) the use of the counterstain Congo Red.

A second problem we encountered with IIF was the fading of the FITC label during the long exposures required for photomicrography. During the early stages of the project, it was virtually impossible to record the fluorescent label, photographically, during a 2 min exposure, due to photobleaching. Subsequently, by using a mounting medium consisting of Moviol mixed with glycerol and the chemical antibleach para-phenylenediamine (Johnson et al., 1981, 1982), the problem of fluorescence fading was eliminated.

Despite the improvements made to the IIF method, its sensitivity in detecting rotavirus was never greater than the SPA or LAB methods, both of which had certain technical advantages over IIF. The SPA method, which makes use of the unique affinity of Staphylococcus aureus Protein A for the Fc-region of immunoglobulins, is relatively inexpensive to prepare. The label is the bacterium itself, which can easily be prepared in any microbiology laboratory. The label is detected by acridine orange staining of the preparation, in combination with ultraviolet or short wavelength blue radiation. Under these conditions the bacterium appears, by light microscopy, as a distinct orange coccus (Goldner et al., 1983). When the SPA method is used to detect SA-11 virus infection in MA-104 cells, the bacterial label attaches to any antiviral antibody that has complexed with intracellular viral antigen. As in the case of the IIF method, it is necessary first to solublize

the plasma membrane with acetone to permit exposure of the antigen to the surface and to enhance passage of the viral antibody to the antigen.

The SPA method was almost as sensitive as the LAB method in detecting rotavirus antigen; after an incubation period of 24 hours it could detect an inoculum of 125 $TCID_{50}$ versus 100 $TCID_{50}$ detected by the LAB method. Furthermore, it was more sensitive than IIF, which could detect only 170 $TCID_{50}$ at 24 hours. Finally, microscopy and photomicrography with SPA were both superior to the other methods, due to the distinctive label and the intensity of the acridine orange stain.

We introduced the labelled avidin-biotin (LAB) method because of its impressive association constant of 10^{-15} M; this is many orders higher than that recorded for antigen-antibody (10^{-5} - 10^{-9}M). Only very recently have avidin and biotin immunoassay reagents been available commercially, but the few publications describing their use report increased sensitivity and decreased reagent requirements. In our study on rotavirus we obtained similar results. By LAB we detected the smallest amount of antigen. When a multiplicity of only 1 $TCID_{50}$ of virus per 25 cells was inoculated into cell cultures, progeny viral antigen could be detected as early as 4 hours p.i. This is particularly impressive when one considers that the earliest detection of rotavirus by electron microscopy, using the same virus-cell system, has been found to be 6 hours (Quan & Doane, 1983).

Conclusions. On the basis of the results obtained from stage 1 of this project, we recommend the use of the LAB method for detecting rotavirus antigen in cell culture. Extending this further, we recommend its use for the rapid detection of any virus that has been isolated in cell culture.

However, like other immunoassays it has one major limitation. Because of the high degree of specificity of the viral antisera used in immunoassays, each virus must be tested for on an individual basis, and heterologous antigens will go undetected. For this reason, we conclude that it is still more appropriate,in general, to identify by electron microscopy viruses isolated in cell culture from environmental samples. As was shown in an earlier study (Doane et al., 1982) a wide range of common viral pathogens can be detected by immunoelectron microscopy (IEM) using human immune serum globulin. Their type identification can then be carried out by IEM, using intersecting antiserum pools.

Future recommendations. On the basis of the results reported here, we recommend that the LAB method now be applied to direct rotavirus detection in environmental samples themselves. Although cell culture isolation procedures currently being used are excellent for amplifying the concentration of many viruses, including animal rotavirus, they will miss human rotavirus, as well as other fastidious viruses such as hepatitis A virus, Norwalk virus, and certain pathogenic strains of adenovirus. Thus the objective should be to reduce the dependancy on cell culture isolation, and to develop methods for direct detection. Two detection methods that should be applied at an early stage are the LAB method, and an ELISA method incorporating avidin and biotin.

As has already been noted, a major factor in favour of using cell cultures is the virus amplification achieved. This is an important consideration, as large quantities of virus are essential if one is to produce reference antisera by immunization of animals, or if epidemiological surveys are to be carried out on the distribution and transmission of a

particular virus. In view of the importance of the human rotavirus as a cause of morbidity and mortality, we consider it to be a top priority to continue with the studies initiated during the past year for the growth of human rotavirus in vitro. Preliminary results are encouraging, and show that at least some strains of the human virus can be induced to grow in vitro.

We further recommend that attention be directed to the detection of Norwalk virus. It has been estimated that approximately 1/3 of epidemics of gastroenteritis are produced by Norwalk virus or closely related viruses (Wolf & Schreiber, 1982). The agent has not yet been propogated definitively in vitro, and is detected in feces by electron microscopy (Kapikian et al., 1972, 1979). Because of the absence of any reference antisera, it will be necessary to rely, initially, entirely on electron microscopy. However, we anticipate that the use of immune serum globulin in combination with electron microscopy will offer a sensitive detection method.

Investigation of methodology for hepatitis A virus is hampered by the high level containment restrictions required for work on this virus. Until such time as the necessary facilities are readily available, it will be difficult to conduct research in this area.

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TABLE 1. Controls Used to Assess the Specific Activity of the Rotavirus - SPA Method

Controls (AO-stained)	MA104 Cells Uninfected	Infected
Cells alone	-*	-
Cells + normal rabbit serum (NRS)	-	-
Cells + anti-NCDV antiserum	-	-
Cells + SPA	-	-
Cells + NRS + SPA	-	-
Cells + pooled human $\gamma\gamma$ + SPA	-	+
Cells + anti-NCDV antiserum + SPA	-	+

* Presence (+) or absence (-) of bacterial cells on cell cultures. SA-11 virus was used at a concentration of 2×10^4 $TCID_{50}$/ml. Infected cultures were examined at 18 hr. post inoculation.

TABLE 2. Controls Used to Assess the Specific Activity of the Rotavirus - IIF Method

Controls	MA104 Cells Uninfected	Infected
Cells alone	- *	-
Cells + NRS	-	-
Cells + anti-NCDV antiserum	-	-
Cells + anti-Ig-FITC	- (bf)	- (bf)
Cells + NRS + anti-Ig-FITC	- (bf)	- (bf)
Cells + pooled human $\gamma\gamma$ + anti-Ig-FITC	- (bf)	- (bf)
Cells + anti-NCDV + anti-Ig-FITC	- (bf)	+ (bf)

* Presence (+) or absence (-) of specific fluorescence.
bf = background fluorescence.
SA11 virus was used at a concentration of 2×10^4 $TCID_{50}$/ml.
Infected cultures were examined at 18 hr. post inoculation.

TABLE 3. Chessboard Titration to Determine the Optimum Concentrations of Rotavirus Antiserum and SPA Cells.

Antiserum Dilution (Reciprocal)	SPA concentration (cells/ml)							
	$3x10^{10}$	$1.5x10^{9}$	$4x10^{7}$	$2x10^{7}$	$1.3x10^{7}$	$1x10^{7}$	$8x10^{6}$	$4x10^{6}$
1	4+*	4+	2+	1+	1+	1+	1+	1+
10	4+	4+	2+	1+	1+	1+	1+	1+
20	4+	4+	2+	1+	1+	1+	1+	1+
40	4+	4+	2+	1+	1+	1+	1+	1+
50	4+	4+	2+	1+	1+	1+	1+	1+
80	4+	2+	1+	1+	0	0	0	0
100	2+	2+	1+	1+	0	0	0	0
200	1+	1+	1+	0	0	0	0	0

* Extent of SPA label recorded on rotavirus-infected cell culture (4+= max.) SPA volume used = 0.1 ml/culture.

TABLE 4. Chessboard Titration to Determine Optimum Incubation Times for Rotavirus Antiserum and SPA Cells.

Antiserum Incubation Time (Minutes) at 37°C	SPA Incubation Time (Minutes) at 37°C							
	5	10	20	30	40	60	80	120
10	0*	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0
30	0	0	1+	1+	1+	1+	1+	1+
40	0	0	1+	4+	4+	4+	4+	4+
60	0	0	1+	4+	4+	4+	4+	4+

*Extent of SPA label recorded on rotavirus-infected cell culture (4+= max.). SPA volume used = 0.1 ml/culture.

TABLE 5. Comparison of Sensitivity in Detection of Rotavirus of Three Different Methods

Dilution of Rotavirus Inoculum	Rotavirus Detection Method		
	CPE	IIF	SPA
10^{-1}	15/15*	5/5	5/5
10^{-2}	15/15	5/5	5/5
10^{-3}	6/15	5/5	5/5
10^{-4}	3/15	4/5	5/5
10^{-5}	3/15	3/5	3/5
10^{-6}	3/15	2/5	3/5
10^{-7}	0/15	0/5	0/5
10^{-8}	0/15	0/5	0/5

Calculation of $TCID_{50}$/ml by Kärber ($\log TCID_{50} = D - \Delta\ (S - 0.5)$):

CPE	IIF	SPA
$\sim 3.2 \times 10^4$	$\sim 2 \times 10^6$	$\sim 2 \times 10^6$

*Proportion of inoculated cultures recorded as positive.

TABLE 6. Controls Used to Assess the Specific Activity of the Rotavirus - LAB Method

Controls	MA104 Cells	
	Uninfected	Infected
Cells + mountant	-*	-
Cells + buffer	-	-
Cells + anti-NCDV antiserum	-	-
Cells + B-Ab	-	-
Cells + Av-FITC	-	-
Cells + anti-NCDV + B-Ab	-	-
Cells + anti-NCDV + Av-FITC	-	-
Cells + B-Ab + Av-FITC	- (bf)	- (bf)
Cells + NRS + B-Ab + Av-FITC	- (bf)	- (bf)
Cells + anti-NCDV + B - Ab + Av-FITC	- (bf)	+ (bf)

* Presence (+) or absence (-) of specific fluorescence.
bf = background fluorescence
B-Ab = biotin-conjugated antibody
Av-FITC = FITC - conjugated avidin
NRS = normal rabbit serum

TABLE 7. Determination of Optimum Concentrations of Biotin-Conjugated Antibody and FITC-Conjugated Avidin for the LAB Rotavirus Method.

Dilution of Biotin-Conjugated Antibody (reciprocal)	Dilution of FITC-Avidin (reciprocal) 1	10	100	1000	2000
4	10/1*	10/1	10/1	10/1	3/.3
32	10/1	10/1	10/1	10/1	3/.3
256	10/1	10/1	10/1	10/1	3/.3
512	10/1	10/1	10/1	10/1	3/.3
1024	10/1	10/1	10/.6	6/.6	3/.3
2048	10/1	10/.6	10/.6	0/0	0/0
4096	0/.3	0/.3	0/.3	0/0	0/0

*S/N ratio (Maximum signal = 10; maximum noise = 1)

TABLE 8. Comparison of IIF, SPA and LAB Rotavirus Detection.

	IIF	SPA	LAB
Earliest detection of antigen p.i. (M.O.I. = 1:25; 2 x 10^4 $TCID_{50}$)	5 hr	5 hr	4 hr
Minimum virus inoculum required ($TCID_{50}$/0.1ml)	170	125	100
Minimum dilution of antiviral antiserum required	1/1260	1/1000	1/1600
Visual quality of signal	3+	4+	3+
Film exposure time required	1-2 min	10-15 sec	1-2 min
Cost	moderate	low	moderate

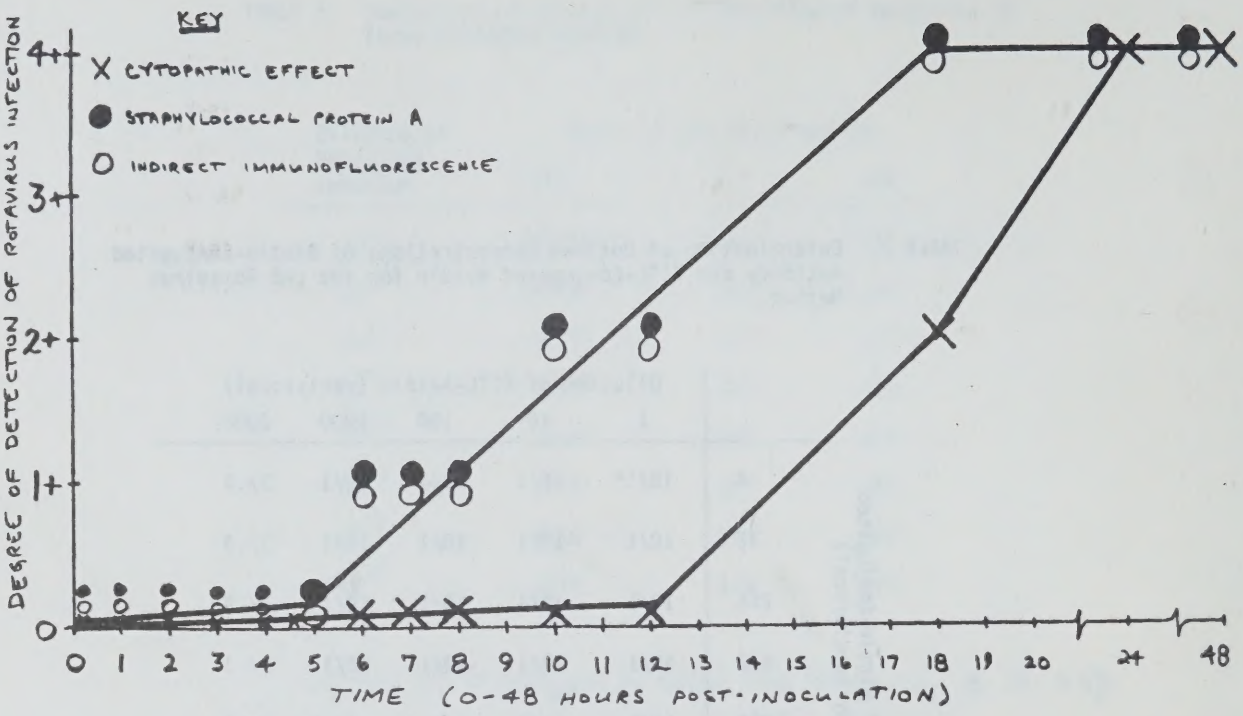

Figure 1. Comparison of the minimum time required for rotavirus detection by three different methods.

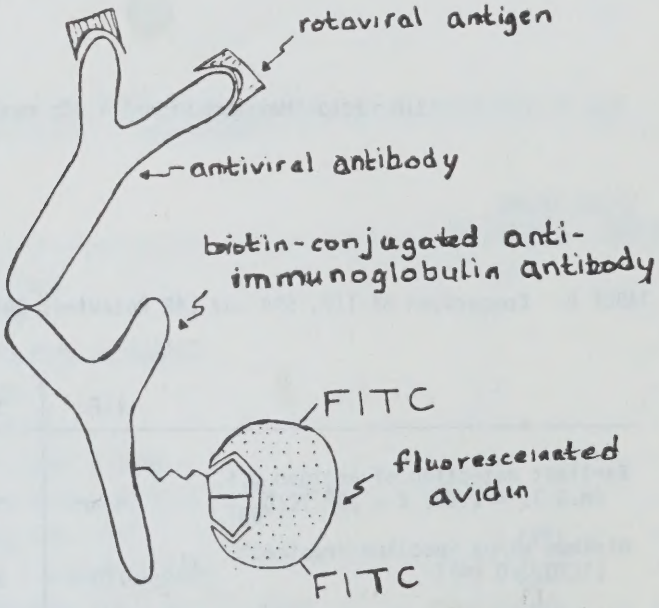

Figure 2. Reactants involved in the LAB method.

THE EPIDEMIOLOGY OF SWIMMING - RELATED ILLNESS AT SELECTED CONSERVATION AREAS

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Abstract

During the summers of 1979, 1980, and 1982 swimmers and non-swimmers were interviewed in prospective cohort epidemiological - microbiological studies carried out at beaches in Ontario. Of more than 8,000 people interviewed, 9.4% of the swimmers became ill versus 3.4 to 3.7% of the non-swimmers. Swimmers experienced respiratory ailments most frequently followed by: gastrointestinal, eye, ear, skin, and allergic symptoms, respectively. Water samples collected at the beaches were routinely analyzed for fecal coliforms, fecal streptococci, total staphylococci, and viruses and, in most surveys, for heterotrophic bacteria and Pseudomonas aeruginosa organisms as well. In 1980, viruses were isolated from the water at two conservation area beaches but not from the water of any of the Great Lakes beaches surveyed. Morbidity among swimmers was shown to be related to staphylococcal counts ($p < 0.0004$) and fecal coliform levels ($p < 0.0058$). Data from a 1983 study which was conducted at Boyd, Claireville, Heart Lake, Kelso and Albion Conservation Areas and at Professors's Lake in Brampton will be used to augment the results from previous surveys.

Introduction

Swimming at public beaches is a very popular pastime in the province of Ontario. Akin et al. (1983) reported that the World Health Organization (1976) revealed that, annually, approximately 500 million people are affected by incapacitating water-borne or water-associated illnesses, and

that about 25% of the world's hospital beds are occupied due to unwholesome water.

In Ontario, the guideline for fecal coliforms, the present recreational water quality indicator of choice in North America is a geometric mean density of 100 bacteria per 100 ml of water (Ontario Ministry of the Environment, 1978). The limit is 200 fecal coliforms per 100 ml of water in most other Canadian provinces and American states (Cabelli et al., 1979; U.S. Environmental Protection Agency, 1976). There is a dearth of epidemiological evidence, however, to support the current recreational water quality guidelines (Cabelli et al., 1979, 1976; Foster et al., 1971). Documentation of swimming-related illness is, however, existent (Brown, 1983; Baron et al., 1982; Cabelli et al., 1982; Koopman et al. 1982; Calderon and Mood, 1981; D'Alessio et al., 1981; Cabelli, 1980; Cabelli et al., 1979; Ktsanes et al., 1979; Seyfried and Fraser, 1978; Ortiz, 1977; Rosenberg et al., 1976; Cabelli et al., 1976, 1975; Bryan et al., 1974; Stevenson, 1953).

As a result of several prospective cohort epidemiological studies, Cabelli et al.(1982); Cabelli,(1980) detected measurable health affects (10/1000 swimmers experienced "highly credible" gastrointestinal symptoms), at Escherichia coli and enterococcal densities as low as 10 organisms per 100 ml of water, when only 10 to 50 ml of water was ingested. These investigators also promoted the enterococci as the optimal recreational water quality indicator.

In our 1980 prospective cohort epidemiological - microbiological survey, a total of 3,967 swimmers and 2,105 non-swimmers were interviewed at 10 beaches in Ontario. Lake water and sediment samples were collected

at the beaches and analyzed for fecal coliforms, fecal streptococci, heterotrophic bacteria, Pseudomonas aeruginosa, and total staphylococci. Bacterial densities were found to be approximately 10 times higher in the sediment than in the corresponding surface water samples.

It was observed in the 1980 study that 77 (3.7%) of the non-swimmers became ill compared to 371 (9.4%) of the swimmers. Because morbidity among swimmers was shown to be related to staphylococcal counts ($p < 0.0004$) and fecal coliform levels ($p < 0.0058$), Brown (1983) has advocated the use of total staphylococci as a bacterial freshwater recreational water quality indicator.

This paper describes the 1982 component of the on-going investigations designed to detect and quantitate swimming-related morbidity, and to evaluate the use of fecal coliforms and total staphylococci, in particular, as bacterial recreational fresh-water quality indicators. In this particular study, limited virological analyses were performed in order to detect viruses in fresh recreational waters.

2. Materials and Methods

i) Epidemiology

A prospective cohort epidemiological investigation was undertaken, from July through August of 1982, at Boyd Conservation Area (north-west of Toronto, and south of Kleinburg) in Ontario. The investigation was modeled upon Cabelli's American studies (Cabelli et al., 1982; Cabelli, 1980; Cabelli et al., 1979, 1975).

More than 1300 persons were interviewed at Boyd Conservation Area beach, on weekends. Weekend sampling was used in order to obtain maximum numbers of beach-going subjects (Cabelli et al., 1982; Cabelli, 1980; Cabelli et al. 1975). A trained interviewer wearing a t-shirt bearing the words: "Health and Water Quality Study" attempted to interview as many beach-goers, as possible. Family units, in contrast to groups of friends, were preferentially selected, in order to facilitate accurate and accessible follow up information. Beach groups of size six or under were considered optimal. During the initial interview, a contact or spokesperson was appointed for each beach group. The telephone number, address, and best time of day to reach the contact person were recorded. In general, the mother of the family, if present, was selected as the contact person because of her knowledge of family morbidity. Subsequently, each member of the individual beach group was interviewed, at the beach.

A copy of the initial interview form (A1) employed at the beach is attached. Follow-up was done exclusively by telephone. A copy of the telephone follow-up form (A2) is also provided. Confidentiality of information was stressed throughout the study. Telephone follow-up was done within five to seven days after the initial

interview, and the period three days after the initial interview day was followed for morbidity development. Three days was the critical interval chosen such that the information would be readily recallable, would not overlap with the next weekend's sampling, and so that illnesses with long term incubation periods would be excluded, due to the possibility of excessive, confounding variables, as well as attempting to avoid detection of person-to-person spread of disease.

ii) Computer Analysis

All the data were coded for record purposes. Data analyses were performed using SAS (Statistical Analysis System) (SAS Institute Inc., 1982). FUNCAT was the technique employed in SAS, to develop the models for total morbidity in swimmers. Basically the predictive model was, as follows, for swimmers:

$$\text{logit } p = \ln \frac{p}{1-p} \quad \frac{(\text{ill})}{(\text{not ill})} = \mu + \alpha + \beta + \gamma \text{ (count)},$$

where μ is the mean of the illness rate or intercept for the model, α and β are binomial factors (of varying number) which significantly altered the model, and γ is the bacterial count (based on the 3 groups used for counts i.e.: 0-150, 151-500, > 500 organisms/100 ml).

iii) Bacteriological Analyses

Surface water samples were collected at Boyd beach, and occassionally at Claireville Conservation Area and Albion Hills Conservation Area (both north-west of Toronto), at a water depth of at least 50 cm, in a location with a high density of swimmers.

Water was analyzed for: total coliforms, fecal coliforms, fecal streptococci, and total staphylococci. All filtrations of appropriately diluted* samples were done using 0.45 µm Gelman filters.

Total coliforms were detected by placing filters upon M-Endo Agar LES (Difco) (51.0g/l to which 20 ml of 95% ethanol had been added). Incubation of plates was done at 35°C for 22 hr $\pm$2 hr. The plates were incubated in a humid atmosphere. Colonies exhibiting green or gold metallic sheens were considered positive.

Fecal coliforms were detected by placing filters upon m-Tec Agar (see A3). The plates were put into a cakette with ice and incubated at 44.5°C for 23$\pm$ 1 hr. Ice allows for the gradual rise of the incubation temperature to44.5°C. After incubation plates were allowed to stand at room temperature for 30 minutes to allow development of the stronger colour reaction by Klebsiella sp. colonies. All yellow or predominently yellow(i.e. yellow-brown, yellow-green) colonies were considered positive.

Total staphylococci were detected by placing filters upon Vogel-Johnson Agar (Difo) with 0.5% sodium pyruvate. Plates were incubated at 35°C for 48 hr. Round, black, shiny colonies were considered positive. Fecal streptococci were detected by placing filters upon m-Enterococcus Agar (Difco) (42.0 g/l). Plates were incubated at 35°C for 48 hr. $\pm$ 3 hr. Red, maroon, or pink colonies were considered positive.

iv) Virological Analyses

Virological analysis was performed twice, due to budget constrictions. The methods are outlined in detail elsewhere (Jenkins and Cherwinsky, 1982). The method is a membrane filtration procedure using 40 l surface water samples. Final indentification was accomplished using electron microscopy.

* Dependent on turbidity.

Results and Discussion

A list of the dates when epidemiological interviewing was carried out at Boyd Conservation Area is given in Table 1. Completed interviews totalled 1366, there were 107 refusals, and 45(3.2%) incompleted interviews due to such problems as wrong telephone number, no telephone, or long distance calls.

A 100% telephone response rate was achieved in the study, if one disregards interviews which were not possible to follow-up. This supports previous findings (Brown, 1983) that in studies of this type, telephone follow-up is preferable to follow-up by mail, or giving forms away at the beach that would subsequently be returned by mail.

In Table 2, the microbiological sampling dates are listed and the geometric means of the total coliform, fecal coliform, fecal streptococci and total staphylococci counts are given. The suggested guidelines for total coliforms, fecal coliforms, and total staphylococci are limits of 1000, 100, and 100, respectively, and a fecal coliform/fecal streptococci ratio of 4 or less (Ontario Ministry of the Environment, 1978; Brown, 1983). At Boyd Conservation Area, on either all sampling days or epidemiological sampling days, the limits were surpassed for fecal coliforms, and total staphylococci, but not for total coliforms (apart from epidemiological sampling days) and the fecal coliform/fecal streptococcal ratio . At Claireville, the limits were exceeded for total coliforms, fecal coliforms, and total staphylococci, but not for the fecal streptococcal ratio . At Albion Hills Conservation Area, none of the limits were surpassed, although sampling was done on only one day. Clearly, Boyd and Claireville display

some evidence of bacterial recreational water pollution, (Claireville generally more than Boyd,) and as such should be monitored closely, for public health purposes.

Reovirus was detected at Boyd Conservation Area on July 25th, 1982 (Table 2). On that day, the total coliform, fecal coliform, and total staphylococcal limits were all exceeded, in contrast to the fecal coliform/fecal streptococcal ratio. Reovirus' role in clinical morbidity has not been clearly established (Jenkins and Cherwinsky, 1982). Virus went undetected when the total staphylococcal count (the only bacterial count done on August 2nd) was 10 or acceptable. Thus, on July 25th, 1982, total coliforms, fecal coliforms, and total staphylococcal counts reflected the virological problem, in some manner.

In this study there were 873 swimmers (64%) and 493 non-swimmers (36%) analyzed. A comparison of the types of illness experienced by the swimmers and non-swimmers is shown in Table 3. The p values provided are from Fisher's 2-tailed test. As may be seen from the table, the crude morbidity rates revealed a statistically significant difference in illness between swimmers and non-swimmers for total morbidity ($p = 0.0000$), gastrointestinal ailments ($p=0.0025$), and "other" ailments ($p=0.0260$), in contrast to ear, eye, respiratory, allergenic, and skin symptoms.

No direct relationship between variability in bacteriological counts and illness was apparent (Tables 2 and 4).

The data were adjusted for individual categories as shown in Table 5, however, no major alterations occurred. Nonetheless, it is necessary to consider the interactions of these factors, using models, to elucidate any effect that the factors might have on the data.

No evidence existed for a relationship between smoking cigarettes and elevation of respiratory morbidity in either swimmers or non-swimmers.

The predictive models for swimmers to become ill (total morbidity) are presented in Tables 6 and 7. For the fecal coliform model, the factors which significantly altered swimmers becoming ill were eating home food (protective) and putting one's head underwater (protective; this may reflect lack of accurate data from the contact person or splashing). The fecal coliform counts did not significantly contribute to the model ($p=0.4573$), however this may be a function of the limited scale of the study.

For the total staphylococcal model, the factors which significantly altered swimmers becoming ill were: eating beach food (unprotective; a risk factor), eating home food (protective), swallowing water (unprotective), and putting one's head underwater (protective-perhaps as explained above). Total staphylococcal counts did not significantly contribute to the model ($p=0.3746$) which may be a function of the limited scale of the project, however their probability for predicting illness is a little better than that for fecal coliforms, given the two models.

Summary

1. Boyd and Claireville Conservation Areas display some evidence of bacterial recreational water pollution, and thus should be monitored routinely.

2. Although sampling was limited, reovirus was detected in the water at Boyd Conservation Area.

3. Swimmers became ill (total morbidity) (9.4%) more than non-swimmers (3.4%) (significant with p = 0.0000 to 4 decimal places). There was also a statistically significant difference between swimmers and non-swimmers for gastrointestinal (p=0.0025) and other ailments (p=0.0260). These statements apply to crude morbidity rates.

4. Predictive models, although based on results from a small sample size, tended to indicate a better dose-response relationship for total staphylococci (at levels higher than 150 organisms/100 mL) than for fecal coliforms. We therefore recommend the use of both total staphylococci and fecal coliforms as bacterial recreational water quality indicators.

5. Future investigators should consider the roles of swallowing water, and eating beach and home foods in studies of this nature.

6. Future studies should investigate the possibility of establishing a viral recreational water quality guideline.

TABLE 1

BOYD CONSERVATION AREA EPIDEMIOLOGICAL
INTERVIEWING DATES

Dates (n = 11 days)
3/7/82
4/7/82
10/7/82
11/7/82
24/7/82
25/7/82
31/7/82
1/8/82
7/8/82
14/8/82
15/8/82

No. of completed interviews = 1366

No. of refused interviews = 107

No. of incompleted interviews due to wrong telephone number, no telephone, or long distance calls = 45.

TABLE 2

i) Boyd Conservation Area Microbiological Data.

Date	Total Coliform Count (per 100 ml)	Fecal Coliform Count (per 100 ml)	Fecal Streptococci Count (per 100 ml)	Total Staphylococci Count (per 100 ml)	Virus Detected
*3/7	190	32	18	190	ND
*4/7	1055	224	102	1000	ND
*10/7	580	430	191	247	ND
*11/7	655	288	368	278	ND
17/7	890	185	110	460	ND
*24/7	1100	325	290	383	ND
*25/7	1233	607	280	623	+ (Reovirus)
*31/7	ND**	ND	ND	143	ND
*1/8	ND	ND	ND	937	ND
2/8	ND	ND	ND	10	-
*7/8	2067	584	257	353	ND
*14/8	4500	2433	533	337	ND
*15/8	2400	1030	780	1010	ND
22/8	330	90	100	72	ND
25/8	ND	ND	ND	623	ND
28/8	300	130	115	33	ND
29/8	100	10	10	72	ND
Overall mean	1185[a] (741)	490[a] (229)[a]	243 (148)	423[a] (240)[a]	ND
Mean for epidemiology days.	1531[a] (1096)[a]	661[a] (398)[a]	313 (219)	500[a] (407)[a]	ND

Bracketed values indicated geometric means.

* = Epidemiological sampling dates.

a = Exceeds limit.

** = Not done.

TABLE 2 (Continued)

ii) Claireville Conservation Area Microbiological Data

Date	Total Coliform Count (per 100 ml)	Fecal Coliform Count (per 100 ml)	Fecal Streptococci Count (per 100 ml)	Total Staphylococci Count (per 100 ml)
3/7	5000	2200	510	ND
4/7	ND	ND	ND	3000
10/7	2000	440	128	513
17/7	8950	520	270	633
24/7	7650	1445	260	367
25/7	4767	1533	830	420
31/7	ND	ND	ND	250
1/8	ND	ND	ND	683
7/8	3133	1000	487	700
14/8	3000	1247	760	427
15/8	2250	1440	910	540
22/8	ND	220	200	92
28/8	690	105	85	40
29/8	100	10	50	73
verall mean	3754[a](2344)[a]	924[a](501)[a]	408 (288)	595[a](347)[a]

Bracketed values indicate geometric means.

a = Exceeds limit.

TABLE 2 (Continued)

iii) Albion Conservation Area Microbiological Data

Date	Total Coliform Count (per 100 ml)	Fecal Coliform Count (per 100 ml)	Fecal Streptococci Count (per 100 ml)	Total Staphylococci Count (per 100 ml)
4/7	20	4	2	45

TABLE 3

CRUDE MORBIDITY RATES

(Symptom rates per 1,000 persons)*

Category of Illness	Number of Swimmers Ill	Number of Non-Swimmers Ill	Fischer's p value to 4 decimals (swimmers versus non-swimmers for actual numbers)
Total	(82/873) 94	(17/493) 34	0.0000**
Gastrointestinal	(23/873) 26	(2/493) 4	0.0025**
Other	(19/873) 22	(3/493) 6	0.0260**
Ear	(6/873) 7	(0/493) 0	0.0931
Eye	(5/873) 6	(0/493) 0	0.1662
Respiratory	(40/873) 46	(15/493) 30	0.1973
Allergy	(4/873) 5	(0/493) 0	0.3032
Skin	(2/873) 2	(0/493) 0	0.5384

Note: Apart from total morbidity, one person may appear in more than one category.

Where: Total illness = all ailments except sunburn
Gastrointestinal = stomachache, or diarrhea, or vomiting
Ear = earache or runny ears
Eye = styes, or red, itchy, or watery eyes
Respiratory = sore throat, or runny or stuffed nose, or fever, cold or cough
Allergy = allergenic itch, or welts or sneezing
Other = ailments other than above and sunburn

* Using rounded whole numbers. ** Significant at p = 0.05 or less

TABLE 4

CRUDE TOTAL MORBIDITY DATA BY DATE

(Boyd Conservation Area)

Date	Swimmers ill (%)	Non-swimmers ill (%)	% Difference
3/7	5/34 (14.7%)	3/71 (4.2%)	10.5%
4/7	8/59 (13.6%)	2/54 (3.7%)	9.9%
10/7	15/70 (21.4%)	1/22 (4.5%)	16.9%
11/7	4/84 (4.8%)	2/32 (6.3%)	-1.5%
24/7	7/100 (7%)	1/26 (3.8%)	3.2%
25/7	7/86 (8.1%)	0/42 (0%)	8.1%
31/7	5/63 (7.9%)	4/55 (7.3%)	0.6%
7/8	4/99 (4.0%)	1/39 (2.6%)	1.4%
10/8	9/94 (9.6%)	1/44 (2.3%)	7.3%
14/8	7/89 (7.9%)	2/58 (3.4%)	4.5%
15/8	11/95 (11.6%)	0/50 (0%)	11.6%

TABLE 5

ADJUSTED MORBIDITY RATES

Type of Illness and Adjustment	Symptom Rates per 1000 persons	
	Swimmer N= 873	Nonswimmer N= 493
All Illnesses		
Unadjusted	94	34
Sex	94	32
Age	92	38
Contact person	94	35
Swam before or after	93	45
Beach food	93	36
Home food	98	32
Socioeconomic status	91	35
Respiratory		
Unadjusted	46	30
Sex	46	28
Age	44	35
Contact person	46	31
Swam before or after	45	41
Beach food	46	32
Home food	49	27
Socioeconomic status	45	31
Cigarette smoker	46	30
Skin		
Unadjusted	2	0
Sex	2	0
Age	2	0
Contact person	2	0
Swam before or after	2	0
Beach food	2	0
Home food	3	0
Socioeconomic status	1	0

TABLE 5 (cont'd)

	Swimmer N= 873	Non-swimmer N= 493
Eye		
Unadjusted	6	0
Sex	6	0
Age	5	0
Contact person	6	0
Swam before or after	5	0
Beach food	6	0
Home food	6	0
Socioeconomic status	6	0
Gastrointestinal		
Unadjusted	26	4
Sex	26	4
Age	25	3
Contact person	27	4
Swam before or after	26	4
Beach food	26	4
Home food	26	4
Socioeconomic status	24	4
Ear		
Unadjusted	7	0
Sex	7	0
Age	7	0
Contact person	7	0
Swam before or after	7	0
Beach food	7	0
Home food	7	0
Socioeconomic status	7	0

TABLE 5 (cont'd)

	Swimmer	Nonswimmer
Allergy		
Unadjusted	5	0
Sex	5	0
Age	5	0
Contact person	5	0
Swam before or after	5	0
Beach food	4	0
Home food	4	0
Socioeconomic status	5	0
Other		
Unadjusted	22	6
Sex	22	6
Age	23	6
Contact person	22	6
Swam before or after	22	7
Beach food	22	7
Home food	23	7
Socioeconomic status	21	6

TABLE 6

PREDICTIVE MODEL FOR SWIMMERS TO BECOME ILL
(USING FECAL COLIFORMS) - BOYD CONSERVATION AREA

$$\text{logit } p = \ln \frac{p}{1-p} = -1.6977 \text{ (intercept, or mean illness rate)}$$

(- 0.5377 (if ate home food)
(or + 0.5377 (if did not eat home food)

(- 0.2800 (if put head underwater)
(or + 0.2800 (if did not put head underwater)

(+ 0.2518 (if fecal coliforms = 0 - 150 per 100 ml. of water)
(or + 0.002804 (if fecal coliforms = 151 - 500 per 100 ml.)
(or - 0.2546 (if fecal coliforms = > 500 per 100 ml.)

1. p = ill.

   1-p is not ill.

2. This model is based upon total morbidity data.

3. In the model the effects of the factors involved are as follows:

Source	X^2 p value
intercept	0.0001
eating home food	0.0009
head underwater	0.0368
fecal coliform counts	0.4929
likelihood ratio	0.4573 (no significant lack of fit).

TABLE 7

PREDICTIVE MODEL FOR SWIMMERS TO BECOME ILL
(USING TOTAL STAPHYLOCOCCI) - BOYD CONSERVATION AREA

$$\text{logit } p = \frac{p}{1-p} = -1.4959 \text{ (intercept, or mean illness rate)}$$

(+ 0.3058 (if ate beachfood)
(or - 0.3058 (if did not eat beach food)

(- 0.5226 (if ate home food)
(or + 0.5226 (if did not eat homefood)

(+ 0.4362 (if swallowed water)
(or - 0.4362 (if did not swallow water)

(- 0.2667 (if put head under water)
(or + 0.2667 (if did not put head under water)

(- 0.1300 (if total staphylococci = 0 -150 per 100 ml. of water)
(or - 0.1038 (if total staphylococci = 151 - 500 per 100 ml.)
(or + 0.2338 (if total staphylococci = > 500 per 100 ml.)

1. p = ill. 1-p = not ill.

2. This model is based upon total morbidity data.

3. In the model, the effects of the factors involved are as follows:

source	X^2 p value	
intercept	0.0001	
eating beach food	0.0107	
eating home food	0.0009	
swallowing water	0.0150	
head underwater	0.0346	
total staphylococcal counts	0.3746	
likelihood ratio	0.2961	(no significant lack of fit).

A1. INITIAL INTERVIEW FORM

DATE: ____ TIME: ____ I.D. ____

LEGEND: YES - 1 NO - 0 DON'T KNOW - 3

I. GIVEN NAMES	1.	2.	3.	4.	5.	6.
II. RELATIONSHIP TO CONTACT PERSON						
III. AGE (Approx. if Adult) & SEX						
IV. SWAM IN PAST 4 DAYS? (Yes or No)						
WHERE AND WHEN?						
HEAD UNDER?						
V. SWAM, OR WILL SWIM TODAY?						
HEAD UNDER?						
VI. SMOKE CIGARETTES?						
under ½ pack daily?						
½ - 1 pack daily?						
more than 1 pack daily?						
VII. SMOKE PIPE? OR CIGAR?						
VIII*. CANCER IN FAMILY?						
TYPE?						
WHAT RELATION?						
XI. CONTACT PERSON ONLY:**						
No. persons in household?						
No. rooms in household?						

PHYSICAL SYMPTOMS ("C" for current; "P" for past week)

1. COLD, UPPER RESPIRATORY						
2. EAR PROBLEMS						
3. STYES OR BOILS						
4. GASTROINTESTINAL						
5. ALLERGIES						
6. SUNBURN						
7. OTHER (NO. & NOTE)						

PERSON'S FULL NAME			
ADDRESS			NOTES
POSTAL CODE			
PHONE NO.			
BEST TIME TO CALL			

* Omitted, eventually due to general distaste expressed

** Due to time factor involved

A2. TELEPHONE FOLLOWUP FORM

DATE: | | | | I.D. | | | | | | | | |

LEGEND: 1 - YES 0 - NO

NAME:

I. Swam on contact day?						
Head under?						
II. Swam in next 3 days?						
Which days? (Circle)						
At this beach only? (Yes or No)						
Head under? (Yes or No)						
III. Eye goggles?						
Ear plugs?						
Nose plugs?						
Swallow any water?						
Eat food bought at beach? (or drink)						
Eat food, at beach, brought from home? (or drink)						

PHYSICAL SYMPTOMS
(Write in date when first noticed)

A. Sore throat						
B. Fever						
C. Cold or cough						
D. Runny or stuffed nose						
E. Earache, Runny Ears						
F. Styes or red, itchy or watery eyes						
G. Stomach ache or nausea						
H. Diarrhea						
I. Vomiting						
J. Boils						
K. Skin rash						
L. Allergenic itch, welts or sneezing						
M. Sunburn: Mild						
N. Moderate						
O. Severe						
P. Other symptoms						
Q. Did you see doctor?						
R. What was his diagnosis?						
S. Did you stay at home because of illness?						
T. How many days?						

If illness serious, may we have your doctor's name and phone number?

A3 m-TEC Agar Formulation

Proteose peptone #3 (Difco)	5.0 g
Yeast extract (Difco)	3.0 g
Lactose (Difco)	10.0 g
NaCl	7.5 g
K_2HPO_4 (BDH)	3.3 g
KH_2PO_4 (BDH)	1.0 g
Sodium lauryl sulphate (Difco)	0.2 g
Sodium desoxycholate (Difco)	0.1 g
Bromocresol purple (BDH)	0.08 g
Bromophenol red (MCB)	0.08 g
Agar (Difco)	15.0 g
Distilled water	1000 ml

- heat to boiling to dissolve agar
- autoclave: 15 mins. at 121^{o}C
- cool to 50^{o}C and dispense in square plates

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THE REMOVAL OF TRIHALOMETHANE PRECURSORS AND SYNTHETIC ORGANIC CHEMICALS FROM POTABLE WATER SUPPLIES BY COAGULATION.

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ABSTRACT

Coagulation is widely used in potable water treatment for the removal of turbidity causing particles. This study investigated the potential of the coagulation process for organic removal, both natural and synthetic. A wide variety of conventional coagulants, recently developed pre-polymerized coagulants and flocculants were studied, with the aid of jar tests.

Results indicate that coagulants are generally highly effective in removing natural organics. Synthetic organics on the other hand do not appear to be removed by coagulation.

As much as 80% of the natural organics present in the water can be removed by coagulation. Consequently, trihalomethane formation, due to chlorination, can be substantially reduced. The operating pH was found to have a significant effect on the removal of organics and also the formation of trihalomethanes. Maximum organic removal typically occurred in the pH range of 5.0 to 6.0. The lowest trihalomethane formation per unit weight of TOC typically occurred at pH 6.0 or below. However, for the aluminum based coagulants, the optimum pH was found to be between 6.0 and 7.0. Above and below these pH's residual aluminum in the water often exceeded the MOE guideline of 0.1 mg/L.

Results of jar tests and filtrability tests showed that the pre-polymerized coagulants both settled and filtered more readily.

INTRODUCTION

In recent years, the removal of organic compounds from potable water supplies has become a matter of great concern to water treatment engineers. Potable waters contain natural organics (compounds such as humic and fulvic acids arising from the breakdown of vegetation). During chlorination, these organics react with chlorine to form trihalomethanes, which are potentially carcinogenic and, therefore, undesirable in potable water. Thus, the removal of natural organics or trihalomethane precursors prior to chlorination would benefit water consumers. Synthetic organic compounds are the other major group of compounds requiring removal. Synthetic organics are compounds manufactured by man and some of these compounds (e.g. the polychlorinated dibenzo-p-dioxins) are highly toxic to life forms. Because they are usually present in low concentration, they are often referred to as micropollutants.

Potable water treatment plants in Canada typically chlorinate their water for disinfection, and coagulate, settle and/or filter their water for turbidity removal. Separate treatment for synthetic organic removal (eg. by activated carbon adsorption) is normally not utilized in Canada. Results of some previous studies indicated that coagulation may have the potential to remove matural organics and synthetic organic chemicals in a cost effective manner (Young and Singer, 1979, Fung 1978). Furthermore, recent advances in coagulation chemistry made available a series of new type of coagulants containing prepolymerized gels believed to be particularly promising in organic removal (Dassonville, 1977/Fiessinger and Bersillon, 1977). This study was

undertaken to try to determine the potential of coagulation for cost effective organics removal. In particular, this study aimed to evaluate the overall organic removal performance of conventional and new coagulants and coagulant aids, and thereby, try to find ways to improve organic removal without the use of an additional treatment step.

COAGULANTS

The most widely used coagulants are based on aluminum or iron salts. New synthetic organic products such as ionic polyelectrolytes are also finding some use.

The most commonly used coagulant in drinking water treatment is alum. Alum is the aluminum sulphate salt, $Al_2(SO_4)_3.18H_2O$. When added to natural water this salt hydrolyzes to the trihydroxide $Al(OH)_3$ which precipitates.

In recent years, several alternative aluminum-based coagulants have been developed in Japan and France. These products have similar principles underlying their design. In France, The Societe Lyonaise des Eaux et de l'Eclairage developed Poly Basic Aluminum Chloride (PBAC) (Fiessinger and Bersillon, 1977). This coagulant is not stable with time, and must be manufactured at the site of its use. The composition of this product is not predetermined, and can be "tailored" according to need, by varying the stoichiometric ratio OH/Al. This value determines the relative amount of different polymeric and monomeric Al-OH species as shown in Figure 1. As the OH/Al ratio increases the size of the Al-OH species increases. Thus for PBAC 1.8, the predominant species

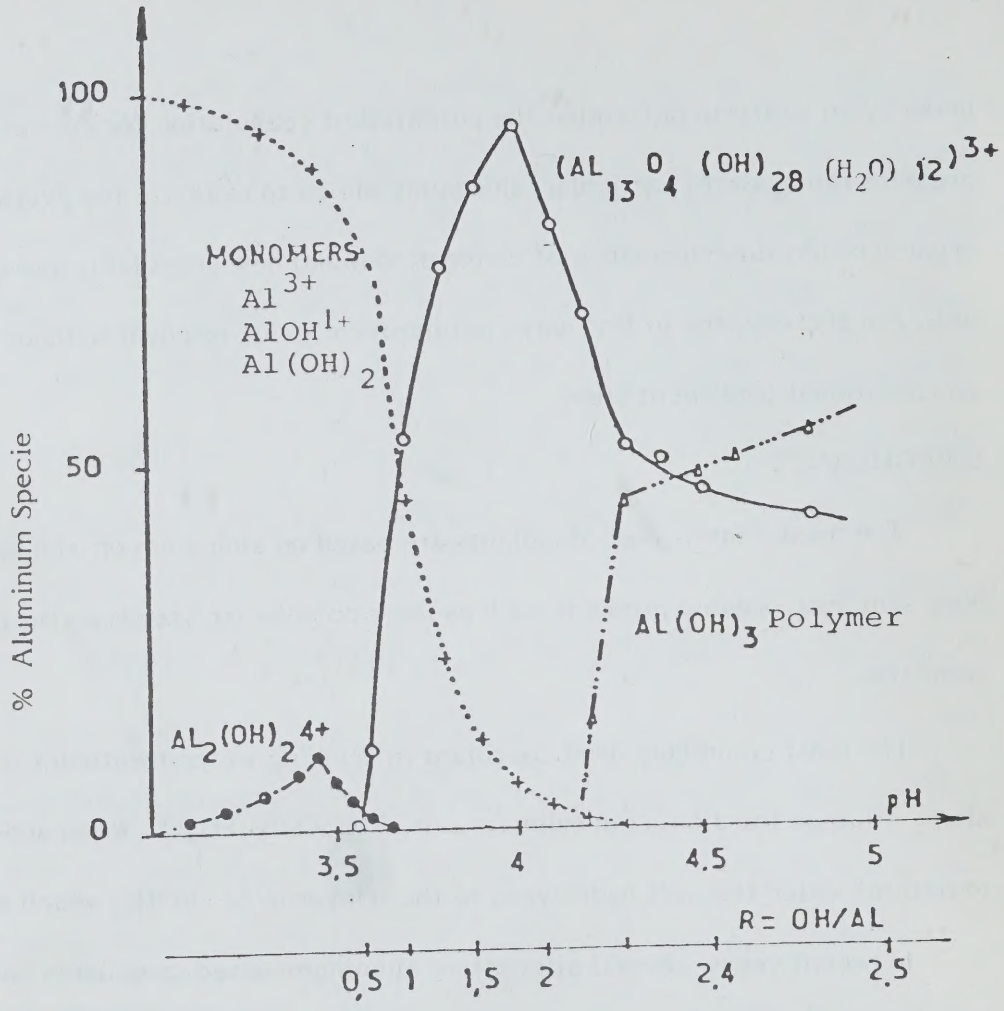


Figure 1 Aluminum speciation as a function of the OH/Al ratio. (Bottero,1980)

is $(Al_{13}O_4(OH)_{28}(H_2O)_{12})^{3+}$ and for PBAC 2.2 , the aluminum atoms are approximately 50% in the $(Al_{13}O_4(OH)_{28}(H_2O)_{12}^{3+}$ form and 50% in the $Al(OH)_3$polymer form. Finally, in the case of PBAC 2.5, the $Al(OH)_3$ polymer becomes the dominant aluminum species.

In Japan, Taki Fertilizer developed Poly Aluminum Chloride Sulfate (PACS) (Taki, 1969) which has the overall composition of $Al(OH)_{1.8}(Cl,SO_4,PO_4)1.2$. The active species is an aluminum hydroxy polymer, stabilized by the presence of the polyanions SO_4 or PO_4. This polyanion enhances the polymerization of the hydroxy polymers without precipitating them. The resulting active species has been found to precipitate quickly with an excess of sulfate which, according to Bersillon et al. (1980), indicates the large size of the polymer. PACS is currently widely used in Japan for the treatment of drinking water.

Ferric chloride is used extensively in wastewater treatment and can be used as an alternative coagulant in drinking water treatment (Carnduff, 1981).

All these coagulants, including the most conventional, i.e., alum, are mineral, and their common property is to hydrolyze to a final product (a trihydroxide) which precipitates, thereby entrapping the particulate material contained in the water.

In addition, organic coagulants have been developed as well. They are polyacrylamides or polyamines, and they are characterized by their charge (anionic, cationic, nonionic), their molecular weight, and their degree of dissociation (Benedek et al., 1977).

METHODOLOGY

In this study organic removals were evaluated with the aid of jar tests (bench-scale coagulation tests) on three typical Ontario water sources, namely:

(i) Lake Ontario water at Hamilton
- typical of Great Lakes water

(ii) Groundhog River water at Fauquier
- typical of water from the Canadian Shield

(iii) Grand River water at Brantford
- typical of water from an industrially polluted sedimentary basin.

Table 1 shows the averaged values of selected physical, chemical and organic parameters for the three raw waters used in this study.

The jar tests were performed with a bench scale flocculator, as described by Benedek (1976). This method allows for the determination of floc settlability in addition to the determination of the physical, chemical and organic characteristics of the treated water. The tests were conducted according to the flow chart shown in Figure 2.

In addition to jar tests, larger-scale batch coagulations, and small-scale continuous filtrability evaluations were performed at a limited number of treatment conditions on Brantford water.

The natural organic concentration was measured as Dissolved Organic Carbon (DOC) on a Dohrman DC54 TOC Analyzer (Dohrman Co., Santa Clara, Calif.) and as Ultraviolet Absorbance at 254nm (A_{254nm}) in quartz cells using an empty

TABLE 1

AVERAGED VALUES OF THE RAW WATER CHARACTERISTICS

	Hamilton	Brantford	Fauquier
pH	7.96	8.48	7.98
Alkalinity mg/L	87.5	250	70.0
Turbidity NTU	0.96	3.30	1.71
UV Abs. (254 nm)	0.300*	0.258**	0.644**
DOC mg/L	2.34	6.44	14.9
TTHMFP ug/L $CHCl_3$	74.4	351	765***

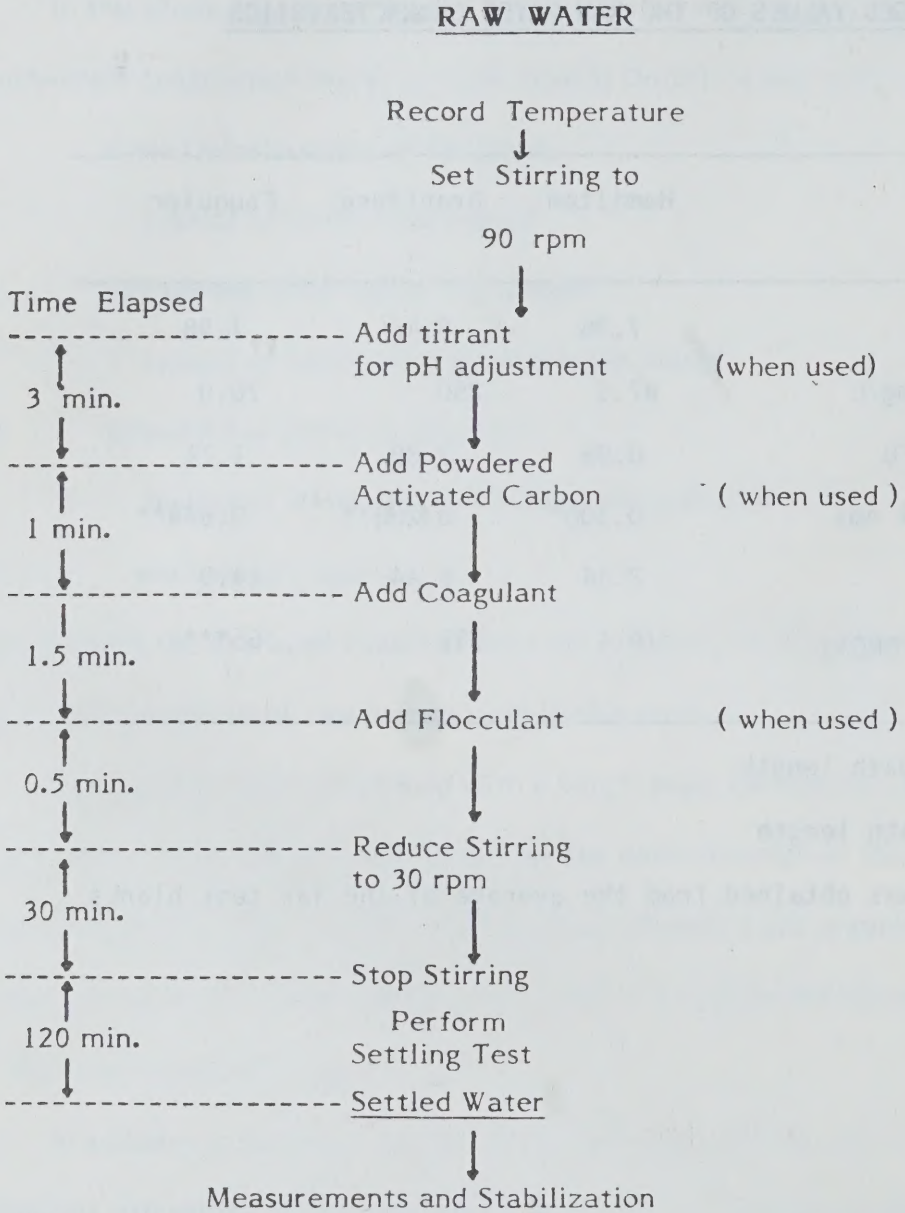
* 10 cm path length

** 1 cm path length

*** value was obtained from the average of the jar test blanks

Figure 2

JAR TEST FLOW CHART



cell as the reference as described by Peel (1980), using a Bausch and Lomb Spectronic 21 Spectraphotometer (Bausch and Lomb Co., Baton, N.Y.).

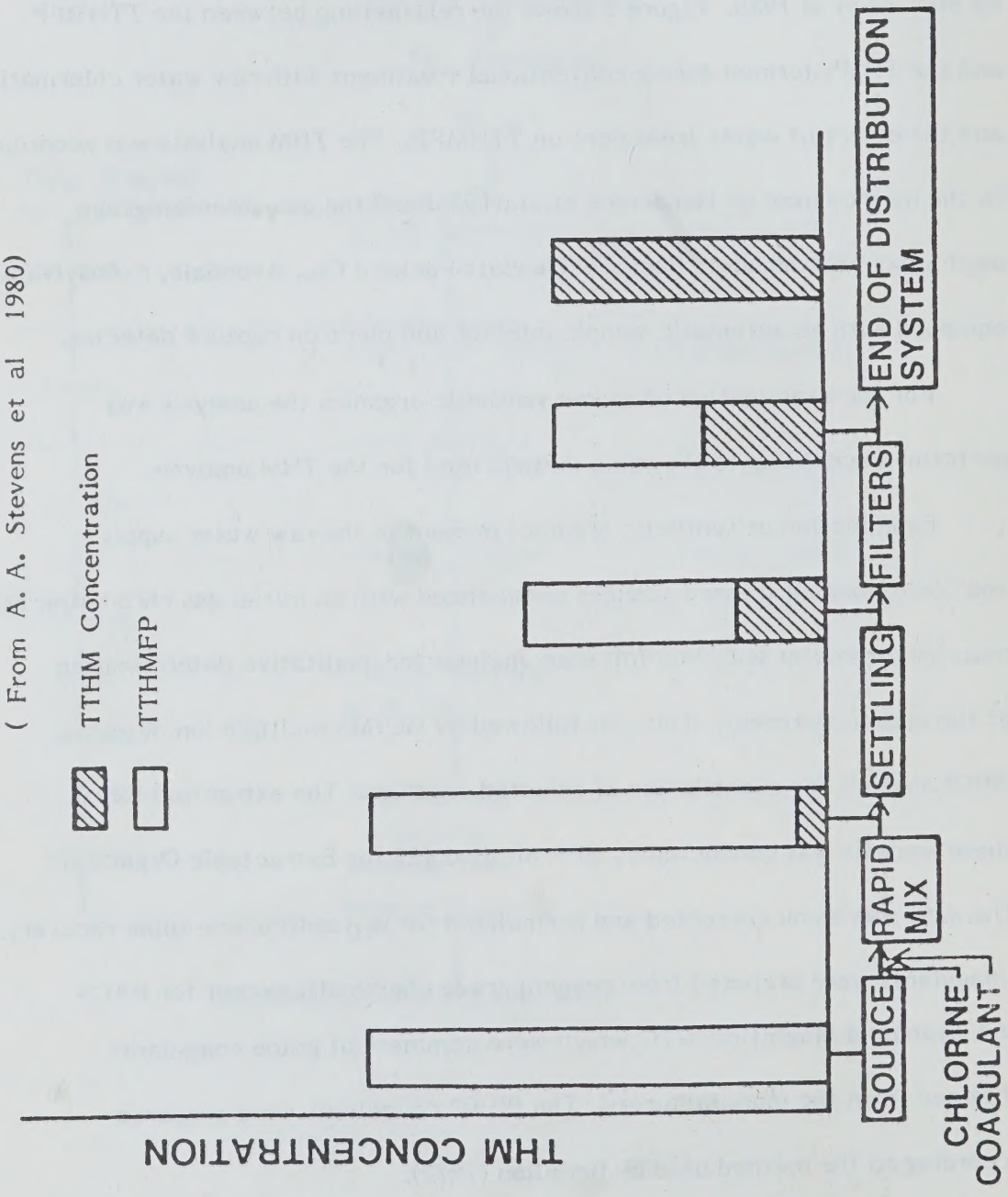
The trihalomethane precursor concentration was evaluated in terms of the Total Trihalomethane Formation Potential (TTHMFP) as described by Stevens et al 1980. Figure 3 shows the relationship between the TTHMFP and the THM's formed during conventional treatment with raw water chlorination and the effect of water treatment on TTHMFP. The THM analysis was according to the method used by Henderson et al.,(1976) and the gas chromatograph used was a 5880 Hewlett Packard (Hewlett Packard Co., Avondale, Pennsylvania) equipped with an automatic sample injector and electron capture detector.

For the examination of spiked synthetic organics the analysis was performed according to the same method used for the THM analysis.

Examination of synthetic organics present in the raw water supply and corresponding treated samples commenced with an initial gas chromatograph/mass spectrometer (GC/MS) full scan analysis for qualitative determination of the organics present. This was followed by GC/MS multiple ion detection (MID) analysis for quantitation of selected organics. The extraction for these analysis was conducted by EPA Method 625 for Extractable Organics. The data was blank corrected and normalized for d_{10} anthracene spike recovery. Coagulants were prepared from reagent grade chemicals, except for PACS and Cyanamid Magnifloc 572C which were commercial grade coagulants obtained from the manufacturers. The PBAC coagulants were prepared according to the method used by Bersillon (1977).

FIGURE 3 TRIHALOMETHANES FORMED DURING CONVENTIONAL TREATMENT WITH RAW WATER CHLORINATION

(From A. A. Stevens et al 1980)



RESULTS & DISCUSSION

ORGANIC PARAMETER CORRELATIONS

Natural organics were measured in this study by Dissolved Organic Carbon (DOC) as well as Ultraviolet Absorbance at 254 nm ($A_{254\ nm}$).

Figures 4 to 6 show the measured DOC as a function of the absorbance at 254 nm, for each water. The path length had to be adjusted as a function of the organic content of the samples. For Hamilton water, a 10 cm path length was adopted, throughout the experiments, whereas the samples from the experiments on Brantford and Fauquier water were measured at a 1 cm path length.

The results show a strong correlation between the UV absorbance at 254 nm and the DOC for Brantford and Fauquier water. This correlation is weaker in the case of Hamilton water.

For each water sampled a UV-DOC correlation curve was constructed and the UV absorbance data was transformed into DOC (mg/L) for further discussion.

When natural organics are chlorinated trihalomethanes are formed. Thus the Total Trihalomethane Formation Potential (TTHMFP) is another measure of the fraction of natural organics that can act as precursors in the trihalomethane formation reaction. A strong correlation was found to exist between the DOC and the TTHMFP for both Brantford and Fauquier water, as shown in Figure 7. When both sets of data are plotted on the same graph, they seem to belong to the same straight line. The interpretation follows:

i) The reactivities of the organic content of Brantford

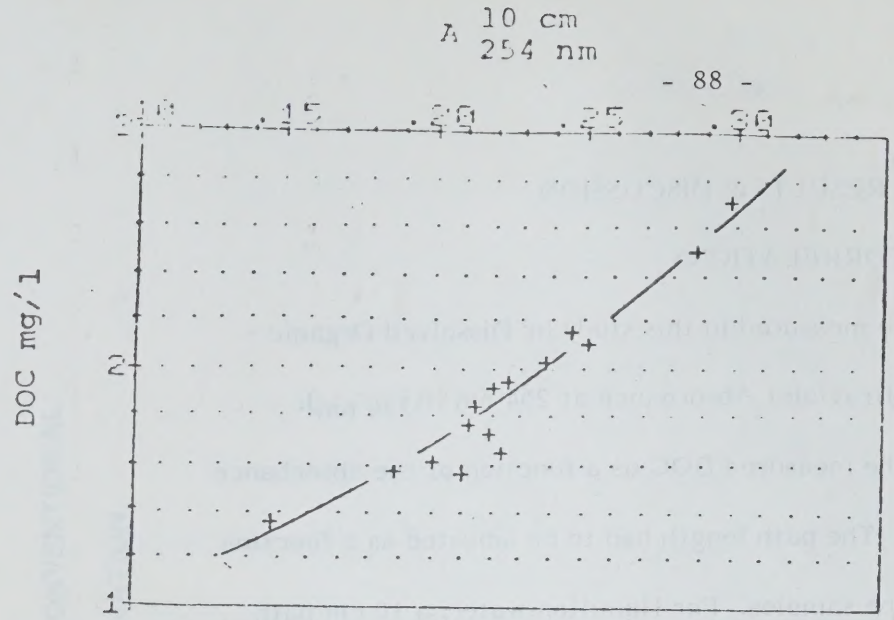


Figure 4
Correlation between UV Absorbance and DOC for Hamilton Water.

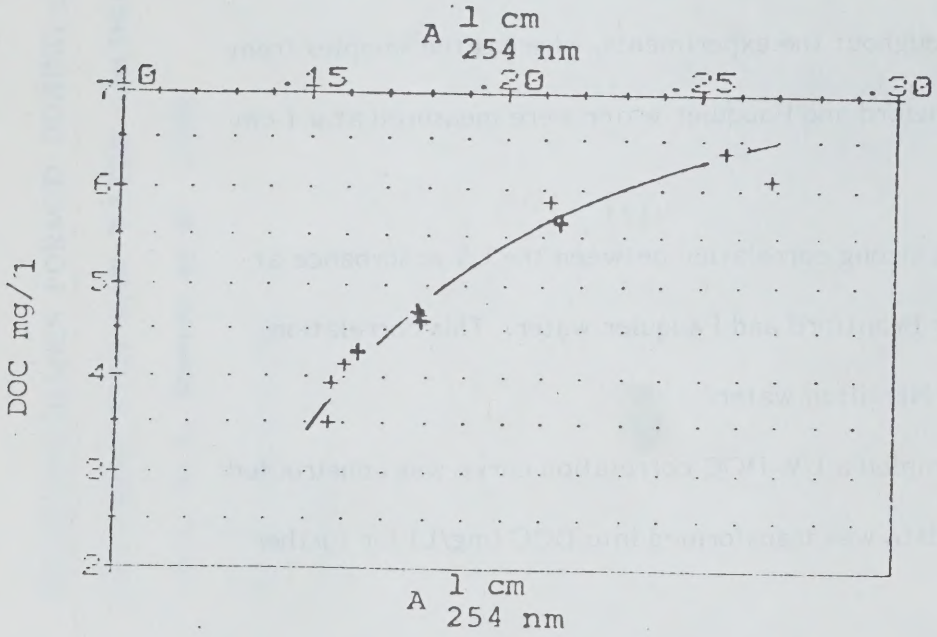


Figure 5
Correlation between UV Absorbance and DOC for Brantford water.

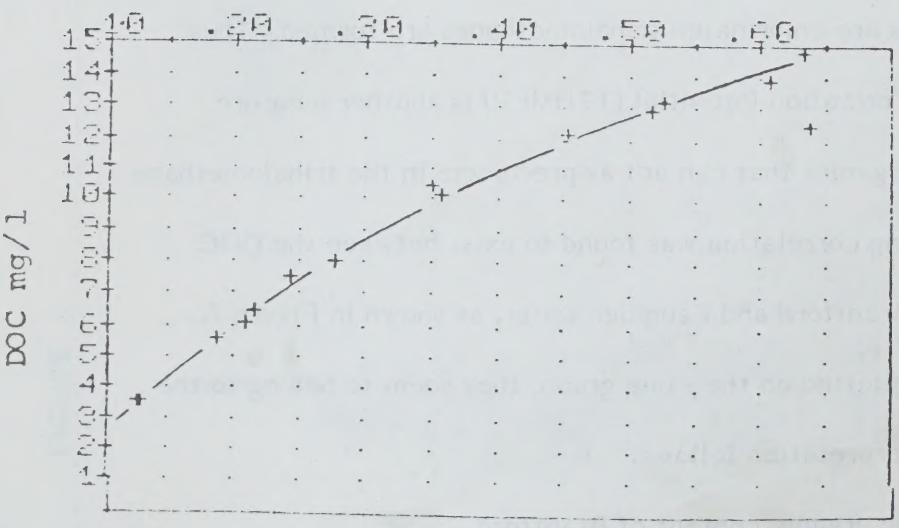
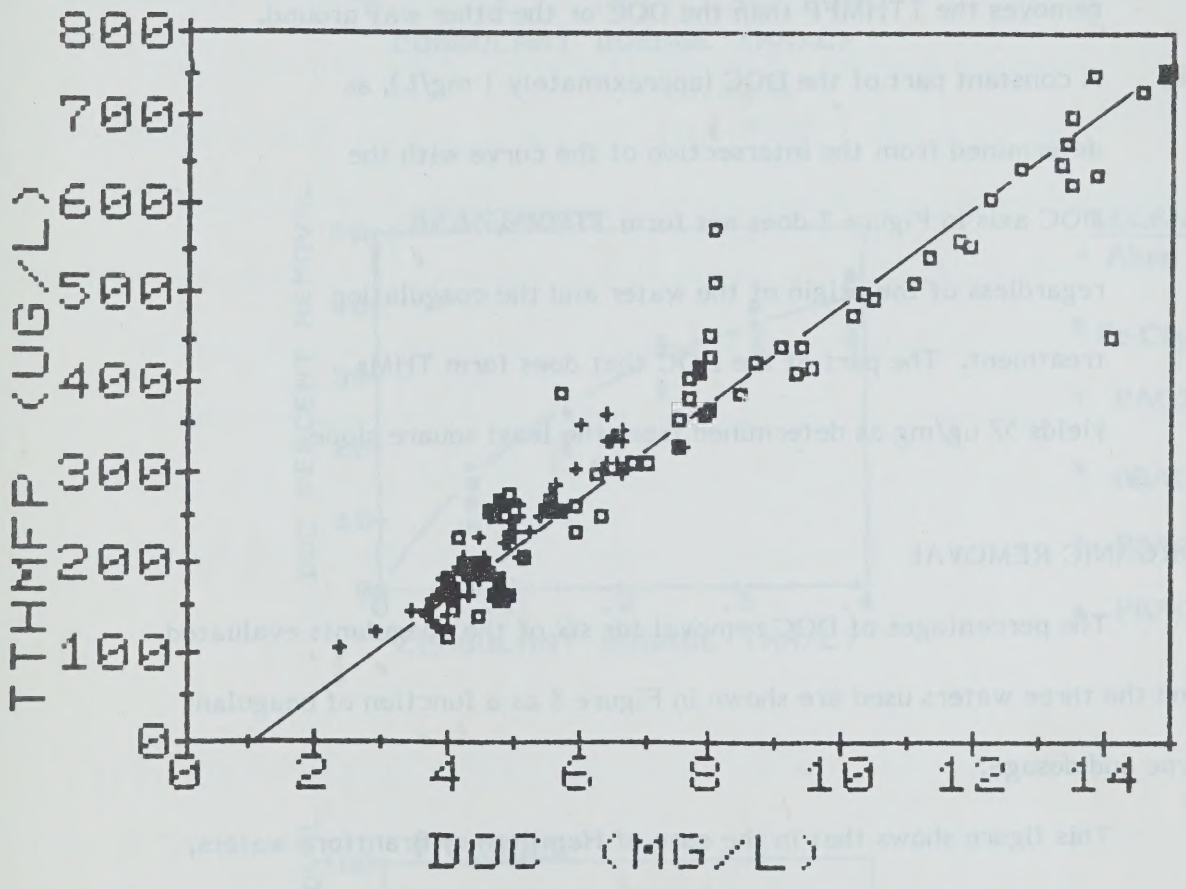


Figure 6
Correlation between UV Absorbance and DOC for Fauquier water.

Figure 7 TTHMFP as a Function of DOC
for Brantford and Fauquier Waters



Legend

+ Brantford Water

□ Fauquier Water

and Fauquier water with regard to chlorine are equal within the experimental conditions

ii) The interaction of the different coagulants with the organic material is not selective, as no coagulant better removes the TTHMFP than the DOC or the other way around.

iii) A constant part of the DOC (approximately 1 mg/L), as determined from the intersection of the curve with the DOC axis in Figure 7 does not form TTHM's, regardless of the origin of the water and the coagulation treatment. The part of the DOC that does form THMs yields 57 ug/mg as determined from the least square slope.

ORGANIC REMOVAL

The percentages of DOC removal for six of the coagulants evaluated and the three waters used are shown in Figure 8 as a function of coagulant type and dosage.

This figure shows that in the case of Hamilton or Brantford waters, the different coagulants have approximately the same effect. On Brantford water, however $FeCl_3$ and alum are slightly more efficient than the Poly-aluminum Chlorides (PACS and PBAC's). This difference in efficiency is enhanced when the coagulants are used on Fauquier raw water. The results

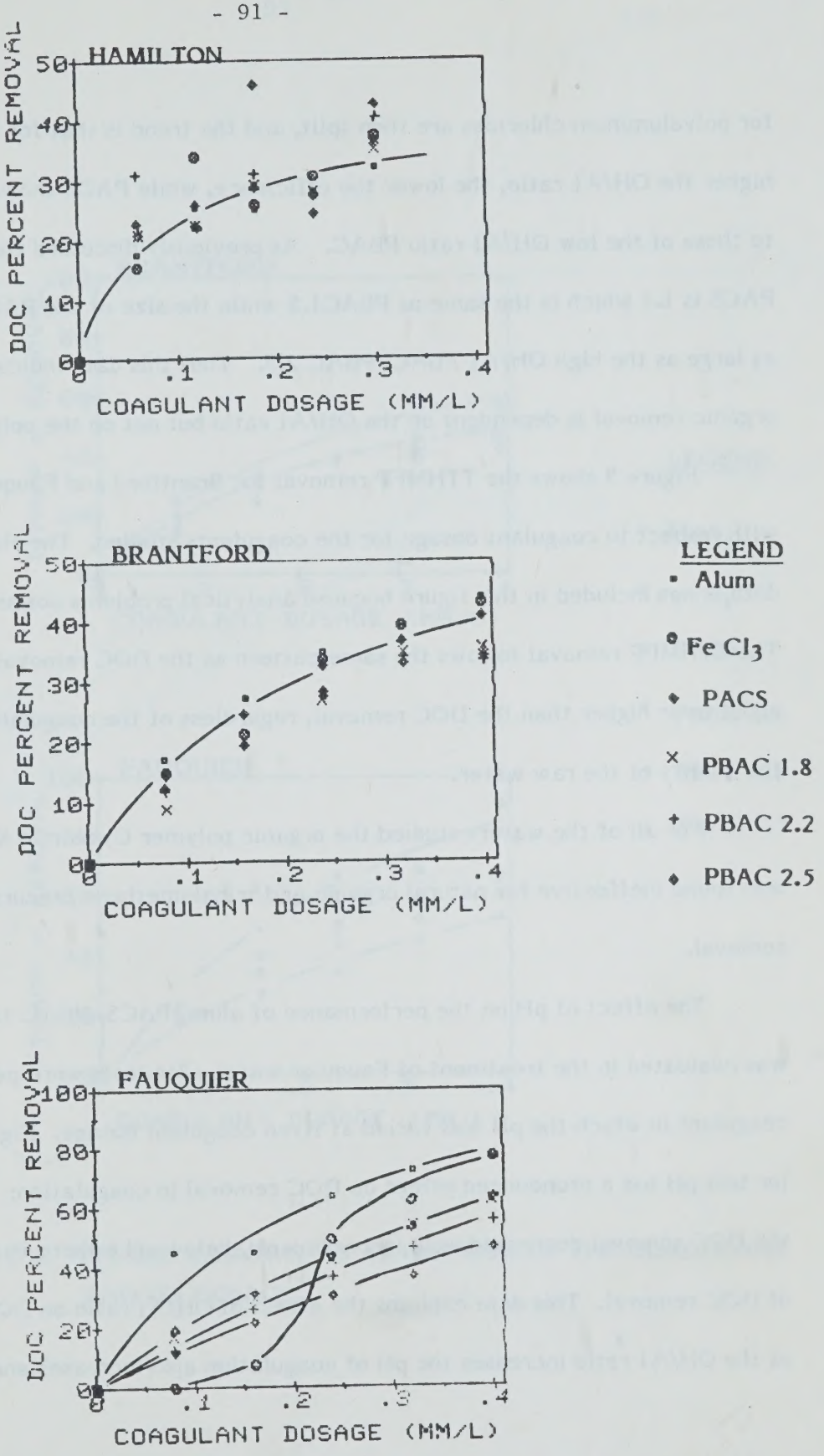


FIGURE 8 DOC REMOVAL AS A FUNCTION OF COAGULANT TYPE AND DOSAGE

for polyaluminum chlorides are then split, and the trend is that for PBAC the higher the OH/Al ratio, the lower the efficiency, while PACS shows removals similar to those of the low OH/Al ratio PBAC. As previously discussed the OH/Al ratio of PACS is 1.8 which is the same as PBAC1.8 while the size of the PACS polymer is as large as the high OH/Al PBAC, PBAC 2.5. Thus this data indicates that natural organic removal is dependent on the OH/Al ratio but not on the polymer size.

Figure 9 shows the TTHMFP removal for Brantford and Fauquier water with respect to coagulant dosage for the coagulants studied. The Hamilton water data is not included in this figure because analytical problems obscured overall trends. The TTHMFP removal follows the same pattern as the DOC removal, and it is equal to or higher than the DOC removal, regardless of the coagulant used or the quality of the raw water.

For all of the waters studied the organic polymer Cyanamid Magnifloc 572C was found ineffective for natural organic and trihalomethane precursor removal.

The effect of pH on the performance of alum, PACS, PBAC 1.8 and $FeCl_3$ was evaluated in the treatment of Fauquier water. Jar tests were performed for each coagulant in which the pH was varied at fixed coagulant dosage. Figure 10 shows that jar test pH has a pronounced effect on DOC removal in coagulation; above pH 6 the DOC removal decreased with increasing pH, below pH 6 there is an apparent plateau of DOC removal. This data explains the effect of OH/Al ratio on DOC removal since as the OH/Al ratio increases the pH of coagulation also increases and thus the DOC

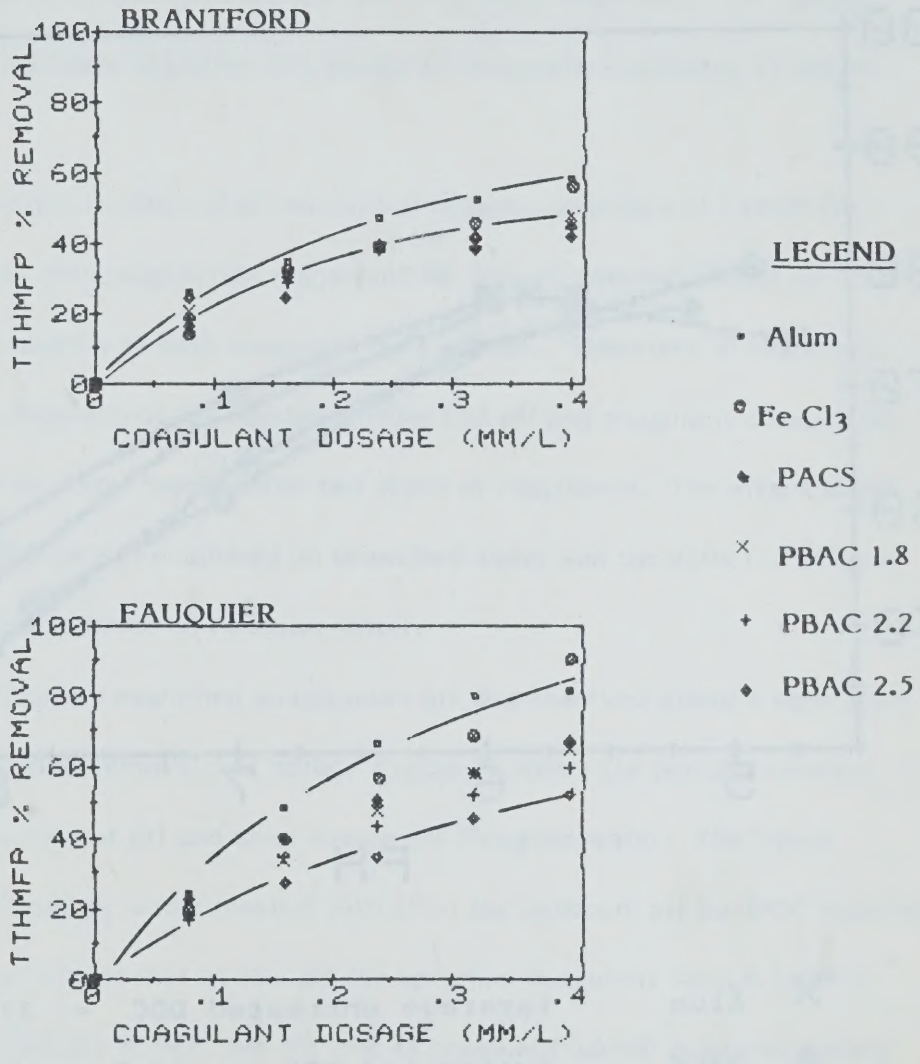
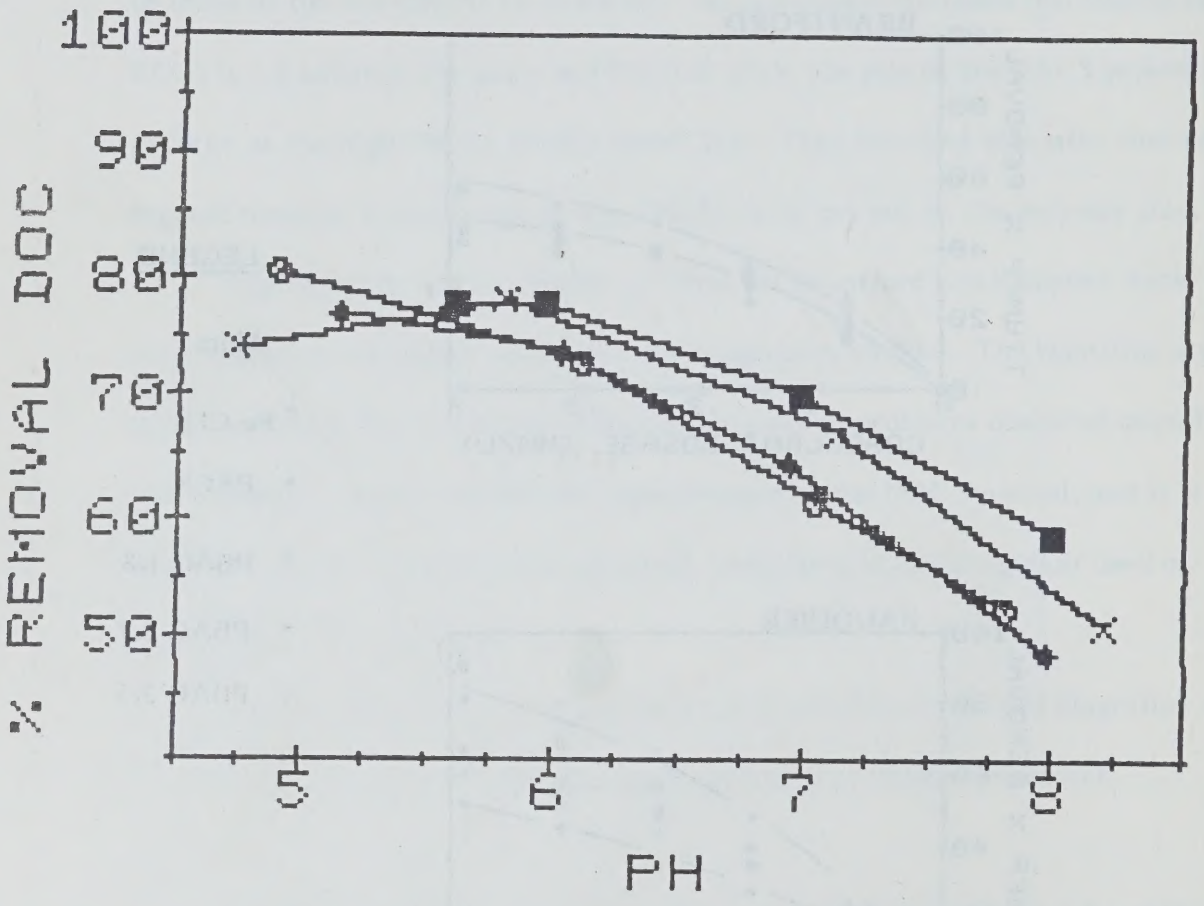


FIGURE 9 TTHMFP REMOVAL AS A FUNCTION OF THE COAGULANT AND ITS DOSAGE.

Figure 10 Percent Removal of DOC as a Function of pH for Fauquier water



Alum (average untreated DOC = 11.6 mg/L)

PACS (untreated DOC = 13.7 mg/L)

PBAC 1.8 (untreated DOC = 13.7 mg/L)

$FeCl_3$ (untreated DOC = 13.6 mg/L)

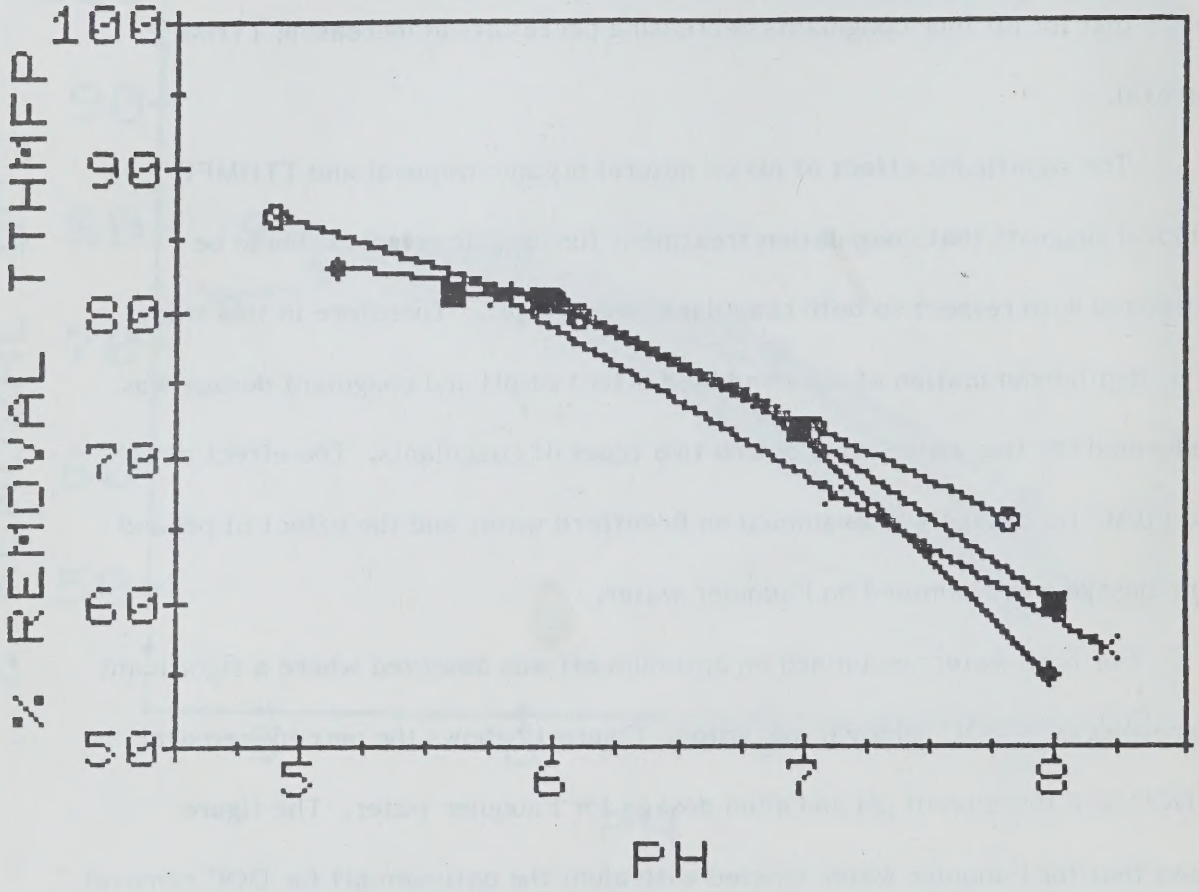
removal per mole of aluminum decreases.

Figure 11 summarizes the percent removal of TTHMFP at the working optimum dosage at various pH's for the four coagulants examined. The results shows that for all four coagulants decreasing pH results in increasing TTHMFP removal.

The significant effect of pH on natural organic removal and TTHMFP removal suggests that coagulation treatment for organic removal should be optimized with respect to both coagulant dose and pH. Therefore in this study an in-depth examination of the combined effect of pH and coagulant dosage was performed for two water supplies and two types of coagulants. The effect of pH and PBAC 1.8 dosage was examined on Brantford water and the effect of pH and alum dosage was examined on Fauquier water.

For both waters examined an optimum pH was observed where a significant improvement in DOC removal was noted. Figure 12 shows the percent removal of DOC as a function of pH and alum dosage for Fauquier water. The figure shows that for Fauquier water treated with alum the optimum pH for DOC removal is approximately 5.7 and that at this pH the optimum coagulant dosage, (where there is a 5% reduction in DOC per 0.1 mM of coagulant added) is approximately 0.20 mM/L. A very similar pattern of DOC removal was observed for Brantford water treated with PBAC 1.8. The optimum pH for this water and coagulant was 6.5 and the optimum coagulant dosage was approximately 0.32 mM/L. The

Figure 11 Percent Removal of TTHMFP as a Function of pH for Fauquier water



X	Alum	(untreated TTHMFP	=	992 ug/L)
✱	PACS	(untreated TTHMFP	=	911 ug/L)
■	PBAC 1.8	(untreated TTHMFP	=	884 ug/L)
⊙	$FeCl_3$	(untreated TTHMFP	=	902 ug/L)

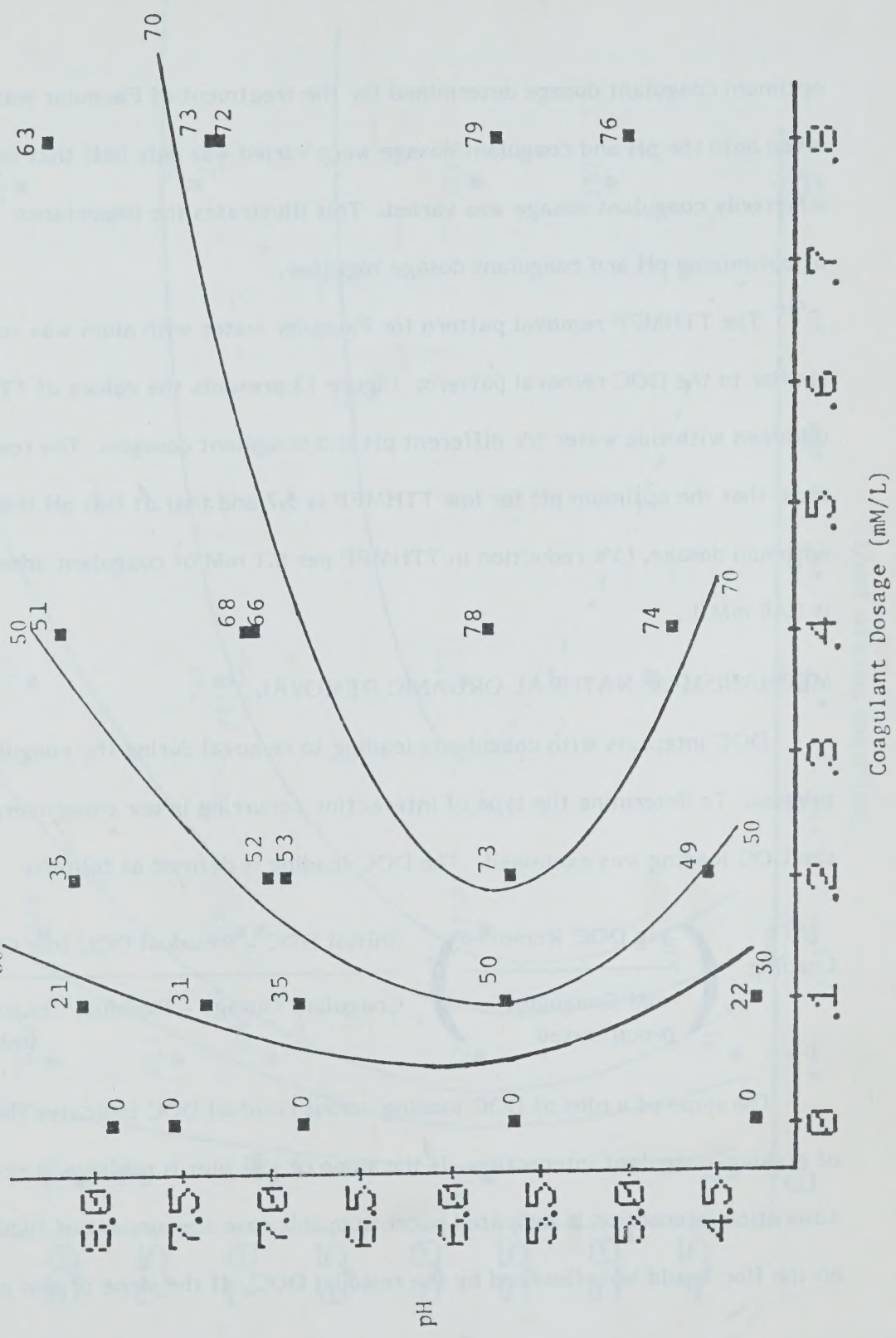


Figure 12 The percent removal of DOC as a function of pH and Alum dosage for Fauquier water (Average untreated DOC = 11.6 mg/L)

optimum coagulant dosage determined for the treatment of Fauquier water when both the pH and coagulant dosage were varied was only half that determined when only coagulant dosage was varied. This illustrates the importance of optimizing pH and coagulant dosage together.

The TTHMFP removal pattern for Fauquier water with alum was very similar to the DOC removal pattern. Figure 13 presents the values of TTHMFP obtained with this water for different pH and coagulant dosages. The results show that the optimum pH for low TTHMFP is 5.7 and that at this pH the optimum dosage, (5% reduction in TTHMFP per 0.1 mM of coagulant added) is 0.40 mM/L.

MECHANISM OF NATURAL ORGANIC REMOVAL

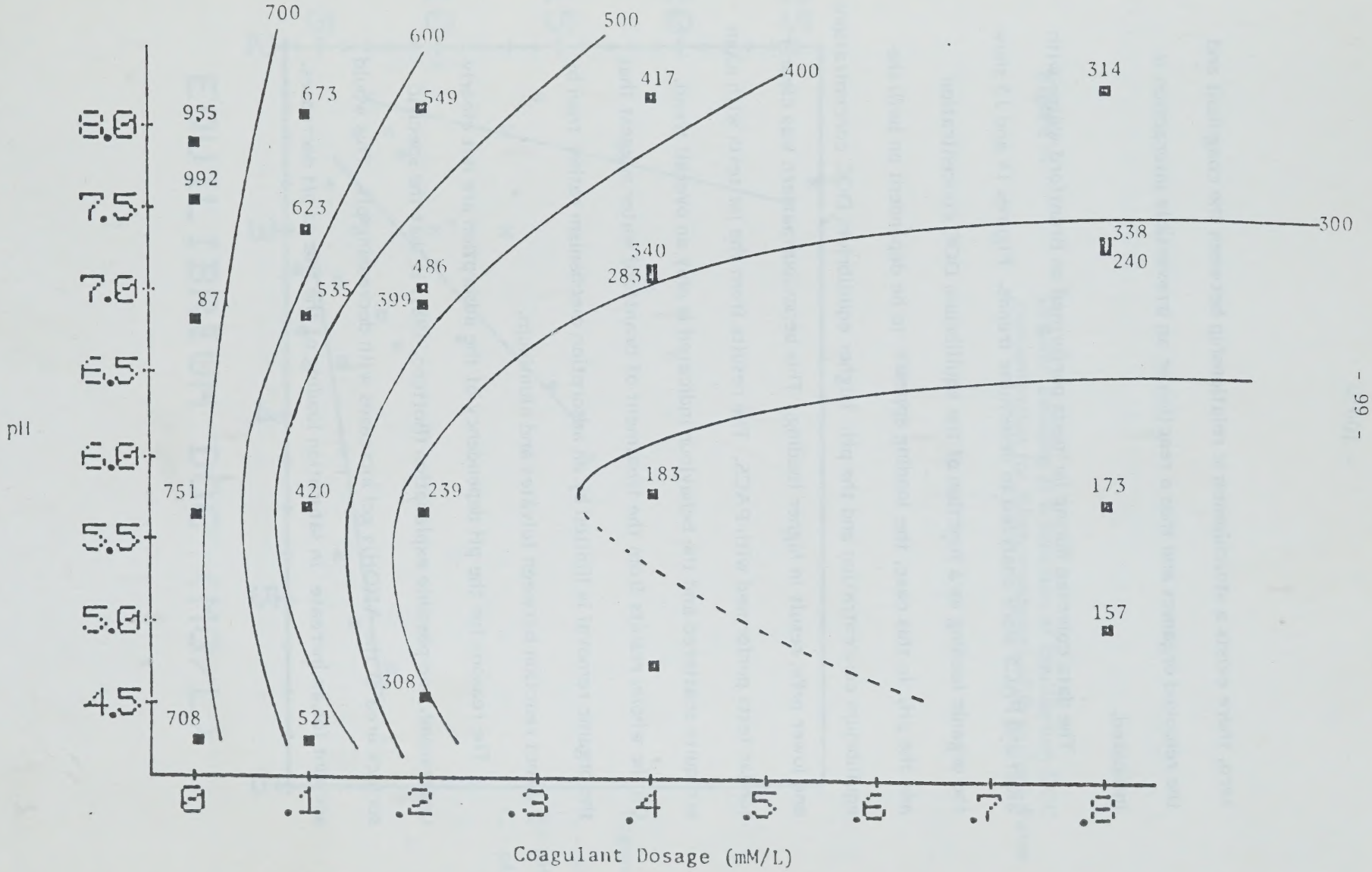
DOC interacts with coagulants leading to removal during the coagulation process. To determine the type of interaction occurring in our experiments, the DOC loading was examined. The DOC loading is defined as follows:

$$\text{Loading} \left(\frac{\text{mg DOC Removed}}{\text{mM Coagulant precipitated}} \right) = \frac{\text{Initial DOC - Residual DOC (mg C/L)}}{\text{Coagulant Dosage - Residual Coagulant (mM/L)}}$$

The slope of a plot of DOC loading versus residual DOC indicates the type of organic-coagulant interaction. If the slope of this plot is positive, a reversible adsorption interaction is indicated because in this case the amount of DOC "loaded" on the floc would be influenced by the residual DOC. If the slope of this plot is

FIGURE - 13

TTHMFP (ug/L) as a function of pH and alum dosage for Fauquier water



zero, there exists a stoichiometric relationship between the coagulant and the removed organics and thus a reaction or an irreversible interaction is indicated.

The data colected during jar tests performed on Brantford water with alum and PACS were analyzed to determine trends. Figures 14 and 15 show the organic loading as a function of the equilibrium DOC concentration and the pH. In this case, the loading appears to be dependent on both the equilibrium concentration and the pH. Higher equilibrium DOC concentrations and lower pH's, result in higher loading. This behaviour pattern was clearer for jar tests performed with PACS. The results from the jar tests with alum are quite scattered and the behaviour indicated is only an overall tread. On the whole, results from the treatment of Brantford water suggest that the organic removal is limited by an adsorption mechanism rather than by a direct reaction between fulvates and aluminum.

The reasons for the pH dependency of the adsorption are not clearly understood, one possible explanation (Bottero, 1981) is that the specific surface area of the $Al(OH)_3$ gel increases with decreasing pH. This would account for an increase in saturation loading of the floc as pH decreases.

Figure 14 Organic Loading as a function of Equilibrium DOC concentration for Alum treatment of Brantford Water

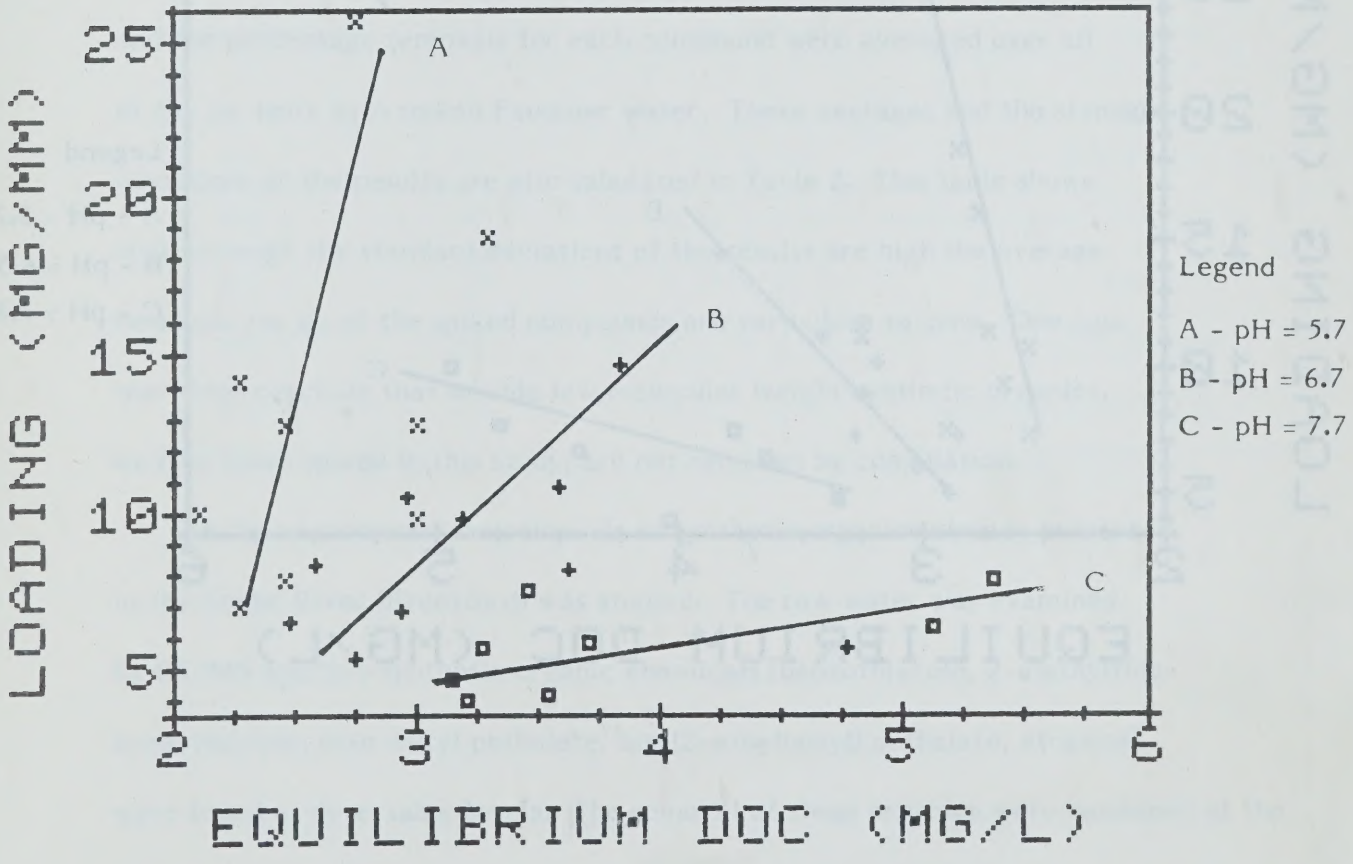
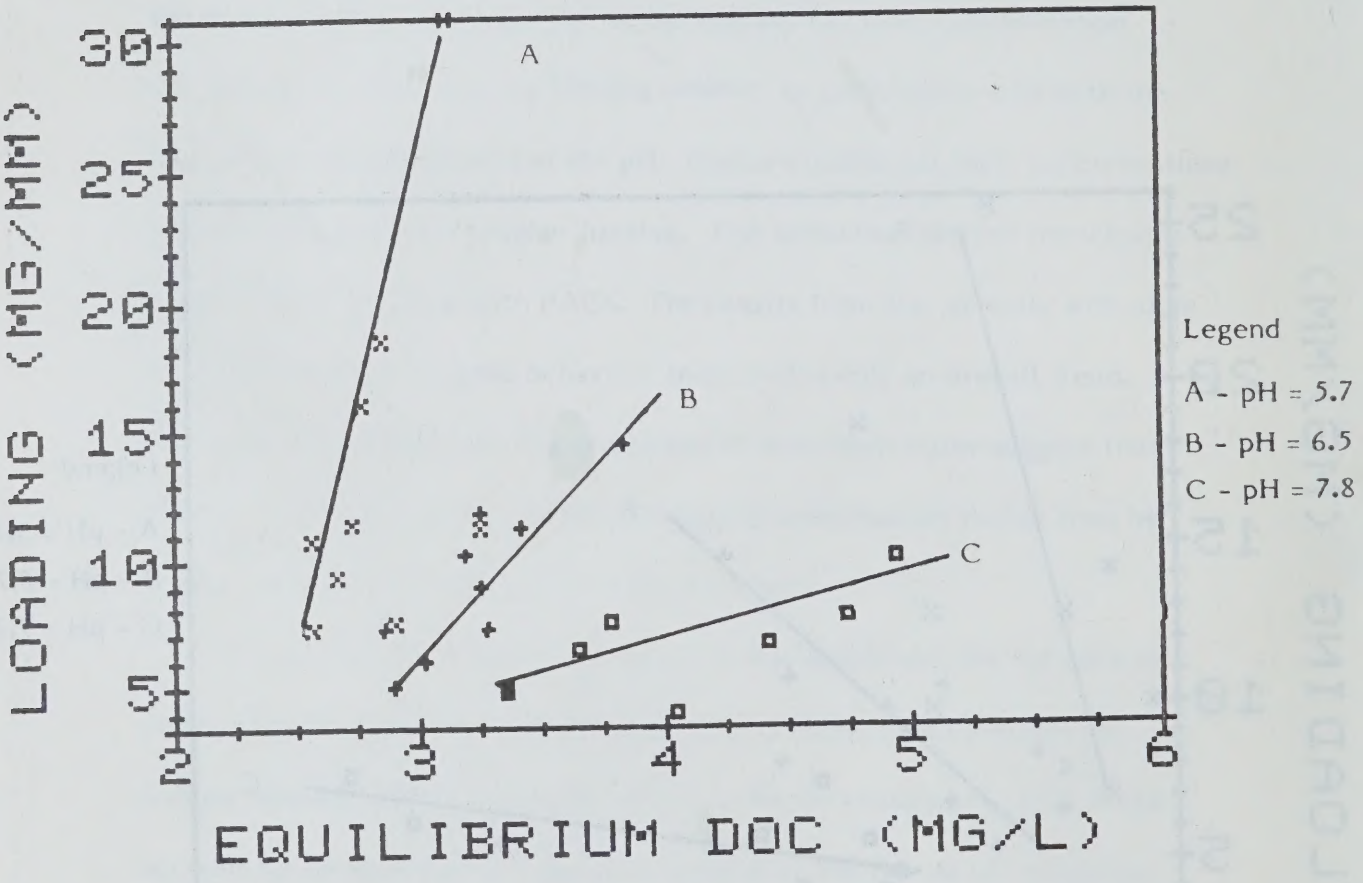


Figure 15 Organic Loading as a function of Equilibrium DOC concentration for PACS treatment of Brantford Water



SYNTHETIC ORGANIC REMOVAL

Two approaches were used to investigate removal of synthetic organics. In the first case selected synthetic organic chemicals (Table 2) were spiked into Fauquier water and their removals evaluated under 28 different pH/alum dosage combinations. Examination of the removal data showed that the percentage removals of the recoverable organics were in general between $\pm$15% and appeared to be random with respect to pH and alum dosage. Thus it was assumed that pH and alum dosage did not affect the observed removals and the percentage removals for each compound were averaged over all of the jar tests with spiked Fauquier water. These averages and the standard deviations of the results are also tabulated in Table 2. This table shows that although the standard deviations of the results are high the average removals for all of the spiked compounds are very close to zero. One can, therefore conclude that soluble low molecular weight synthetic organics, such as those spiked in this study, are not removed by coagulation.

In the second case the removals of synthetic organics already present in the Grand River (Brantford) was studied. The raw water was examined by GC/MS and five synthetic organic chemicals [benzothiazole, 2-methylthio-benzothiazole, di-n-butyl phthalate, bis-(2-ethylhexyl) phthalate, atrazine] were found at detectable levels. The removal of these organics were examined at the

TABLE 2

AVERAGED PERCENTAGE REMOVALS OF SPIKED ORGANICS IN FAUQUIER WATER

Compound	Average Percentage Removal ± Standard Deviation	Number of Points	Analytical Reproducibility
Chloroform	2.2 ± 12.1	28	± 10%
1,1,2-Trichloroethane	-1.6 ± 14.6	28	± 10%
Toluene	1.8 ± 11.8	22	± 10%
Naphthalene	1.6 ± 9.8	22	± 10%
2,4-Dichlorophenol	-3.6 ± 27.0	27	± 10%

optimum treatment conditions with alum and with PACS. In this case variability in chemical analysis (due to limitations in current analytical techniques), and matrix changes (caused by coagulation affecting analytical recoveries) may have obscured small removals. The analytical variability ranged from $\pm$ 21% for atrazine to $\pm$ 74% for bis-(2-ethylhexyl) phthalate. Removals of these synthetic organics at levels greater than the analytical variability were not observed.

OPTIMUM ORGANIC REMOVAL

So far as natural organic and TTHMFP removal is concerned this study has shown that there is no true optimum coagulant dosage and pH, since organic removal continuously increases with increasing dosage and increases with decreasing pH. Potentially, coagulant dosage may be adjusted to reach a predetermined residual organic concentration. Alternatively, the dosage for which an additional increment of coagulant would lead to marginal improvement of the organic residual can be defined as the optimum (for this study this was defined as the dosage at which 0.1 mM/L of additional coagulant resulted in less than 5% reduction of residual organics).

Furthermore, there are other operational factors which must be taken into account in the coagulation step, such as the settling velocity of the floc, the residual turbidity in the clarifier overflow and the residual coagulant concentration.

These parameters were evaluated in the jar tests together with the organic and TTHMFP removals. They were taken into account indirectly by setting maximum or minumum acceptable values based on regulatory guidelines or limitations based on water treatment plant operations. The working criteria used are summarized in Table 3. In general it was found that in the pH and coagulant dosage range for which organic removal was optimized all coagulants removed the turbidity satisfactorily. However, the flocs produced by the prepolymerized coagulants (PACS and PBAC) were easier to remove by settling and also by filtration.

Results consistently indicate that careful attention should be given to the operating pH during coagulation as it both determines the amount of residual aluminum and significantly affects the extent of organic and TTHMFP removal. The coagulation step should be performed at a pH within the minimum solubility band of the aluminum ion, to avoid problems in the distribution network, due to delayed precipitation.

Performance evaluation of four of the coagulants, (alum, $FeCl_3$, PACS and PBAC 1.8) on Fauquier water sampled in one season, resulted in selection of alum and PACS for more intensive study on Grand River water. Both of these coagulants, when used on Fauquier water at pH 6.0 and a dosage of 0.4 mM/L, achieved good natural organic removal (as measured by Dissolved Organic Carbon) and trihalomethane precursor removal (as measured by TTHMFP); residual turbidity, settling velocity and residual aluminum were all acceptable. Furthermore, both coagulants removed approximately 25% more of the natural organics and TTHMFP

TABLE 3

Working Criteria Used to Evaluate
Coagulant Performance

	Maximum	Minimum
pH	8.5	6.5
Residual Aluminum (mg/L)	0.10	--
Residual Iron (mg/L)	0.30	--
Settling Velocity m/h	--	0.2
Residual Turbidity (NTU)	5	--

than is being currently removed at the Fauquier water treatment plant. To obtain these improved results, coagulant dosage at the plant would have to be approximately doubled and the pH of the water adjusted before and after treatment.

Studies were conducted on Brantford water sampled in three different seasons. The optimum dosage of alum or PACS ranged between 0.24 mM/L and 0.30 mM/L depending on the season, and the optimum pH ranged between 6.5 and 6.9. Under these conditions coagulation resulted in up to 47% more removal of the DOC and up to 78% more of the TTHMFP than the coagulation treatment currently being used at the Brantford water treatment plant; residual turbidity, settling velocity and residual aluminum were all acceptable. With respect to organic removal, PACS performed marginally better than alum. However, the settling velocities and residual turbidities in water treated with PACS were improved relative to the values obtained with alum treatment. To obtain these improved organic removals, a coagulant dosage 5 to 6 times greater than the alum dosage currently applied at Brantford would have to be used and the pH of the water would have to be adjusted before and after coagulation.

CONCLUSIONS

i) Coagulation can be a very efficient method for the removal of the natural organic material and for the removal of trihalomethane precursors.

ii) Coagulation treatment conditions can be optimized, with respect to both pH and coagulant dosage, to improve natural organic and trihalomethane precursor removal.

iii) Coagulation was found to be ineffective for the removal of soluble synthetic organic compounds spiked into a raw water. In the case of synthetic organics already present in a raw water, analytical limitations did not allow a clear indication of removal effectiveness (despite the used of surrogate standards to account for method variability). However, removals due to coagulation of these synthetic organics at levels greater than the analytical variability were not observed.

iv) The operating mechanism for natural organic removal during the coagulation/flocculation process appears to be adsorption.

v) The role of pH is extremely important in the definition of optimum treatment conditions for inorganic coagulants as it significantly affects the extent of organic removal, trihalomethane precursor removal, and residual coagulant aluminum or iron concentration. Adjustment in pH alone, regardless of coagulant

dosage can significantly improve organic removal and trihalomethane precursor removal.

vi) The three alternative inorganic coagulants examined (PACS, PBACS and ferric chloride) have similar removal efficiencies at optimum treatment conditions to the conventional coagulant, alum, for removal of organics and THM precursors. However, PACS produced a floc with better settling characteristics and resulted in a lower residual turbidity.

vii) The organic polymeric coagulant (Cyanamid Magnifloc 572C) was not effective for removing organics or THM precursors.

viii) For improvement in the removal of natural organics and the reduction of TTHMFP in waters with high natural organic concentration, and low alkalinity, of which Fauquier water is typical, coagulation can be optimized without dramatically increasing the amount of chemicals required over those already used.

ix) For significant improvement in the removal of natural organics and the reduction of TTHMFP in waters with a moderate natural organic concentration, and high alkalinities, of which Brantford water is typical,significant increases are required in the amounts of coagulants used over the amounts currently used.

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ABSTRACT

A full-scale gravity flow UV system which consists of a series of modules forming two separate rectangular matrices of UV lamps was placed in each effluent channel of a conventional secondary sewage treatment plant.

Photoreactivation and dark repair experiments showed that 3h produced the maximum number of total and fecal coliforms whereas fecal Streptococci showed no increase.

Routine bacteriological sampling has shown that the geometric mean of the total and fecal coliform bacteria before and after photoreactivation can be maintained below the limits of 2,500 and 200 organisms per 100 ml respectively in between the matrices as well as after the two UV units. The chlorinated effluent was similar bacteriologically except for the resistant spore-forming bacteria, Clostridium perfringens, and bacteriophage of Escherichia coli C which were more sensitive to UV irradiation.

A fish toxicity evaluation conducted in the receiving stream showed that chlorinated effluent was toxic to rainbow trout whereas UV irradiated effluent was not.

A cost comparison has shown that UV disinfection is a cost effective alternative to other forms of wastewater disinfection such as chlorination, chlorinaton-dechlorination and ozonation.

The UV and chlorinated effluents have been tested for the following pathogens: Pseudomonas aeruginosa, Yersinia enterocolitica, Campylobacter jejuni and Salmonella sp.

ULTRAVIOLET DISINFECTION OF SECONDARY SEWAGE EFFLUENT

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The elimination of pathogenic microorganisms from wastewater before discharge to receiving waters which may be used for human or livestock purposes is of the utmost importance. In North America, disinfection of treated sewage is accomplished almost exclusively with chlorine. An increasing body of research[1,2,3,4] reveals that chlorine residuals in receiving waters are deleterious to the aquatic biota. Chlorination also produces chlorinated compounds that may be toxic to humans and animals[4]. An alternative to chlorination is ultraviolet (UV) disinfection[5].

To date, studies involving UV disinfection of wastewater have involved the assessment of disinfection efficiency, those factors affecting this efficiency and the outlining of some of the problems with the application of UV irradiation. Laboratory studies with UV light have demonstrated an adequate reduction in indicator bacteria from secondary effluents[6,7,8]. UV irradiated effluent exhibited no toxicity to fish when compared to undisinfected wastewater[3,8]. Jolley et al.[9] showed that UV irradiation of nonvolatile organics in secondary effluent produced only slight chemical changes in the nonvolatile organics while in one effluent mutagenic constituents were eliminated.

Pilot[10,11,12,13,14] and full scale[15] studies of the UV disinfection of sewage effluent have demonstrated that the process can consistently achieve the objective of 200 fecal coliforms per 100 ml of effluent. UV devices designed for

disinfection of potable water were employed in most of these studies.

This project was initiated to assess all the operational aspects, and to monitor the efficiency of a full-scale ultraviolet device which was designed specifically for disinfecting wastewater.

MATERIALS AND METHODS

Project Site Description

The facility chosen for this study is located in Tillsonburg, Ontario, Canada. It is a conventional secondary plant which produces an above average effluent from primarily domestic sewage (Figure 1). The design capacity of the plant is 8.2×10^3 m³/d with an average yearly flow of 4.6×10^3 m³/d. Separate clarifiers, effluent channels and chlorine contact chambers allowed the simultaneous study of UV and chlorine disinfection.

Description of UV Unit

A modular system was designed to provide low maintenance and remain versatile. As shown in Figure 2, the system consists of a series of modules each of which is four quartz sheath enclosed UV lamps (13.8 W of 253.7 nm wavelength light, 76.2 cm arc length/lamp). Two sets of 10 modules for a total of 80 lamps are located in one effluent channel and are hereon described as Unit A. The second channel is 40 cm wider and contains 2 sets of 15 modules for a total of 120 lamps and is hereon called Unit B. The average flow over the lamps of Units A and B are 0.03 m³/sec and 0.02 m³/sec, respectively. Pumps and related maintenance are obviated because it is a gravity flow system. All of the major components are modular thereby eliminating a major shutdown if one component requires maintenance or repairing.

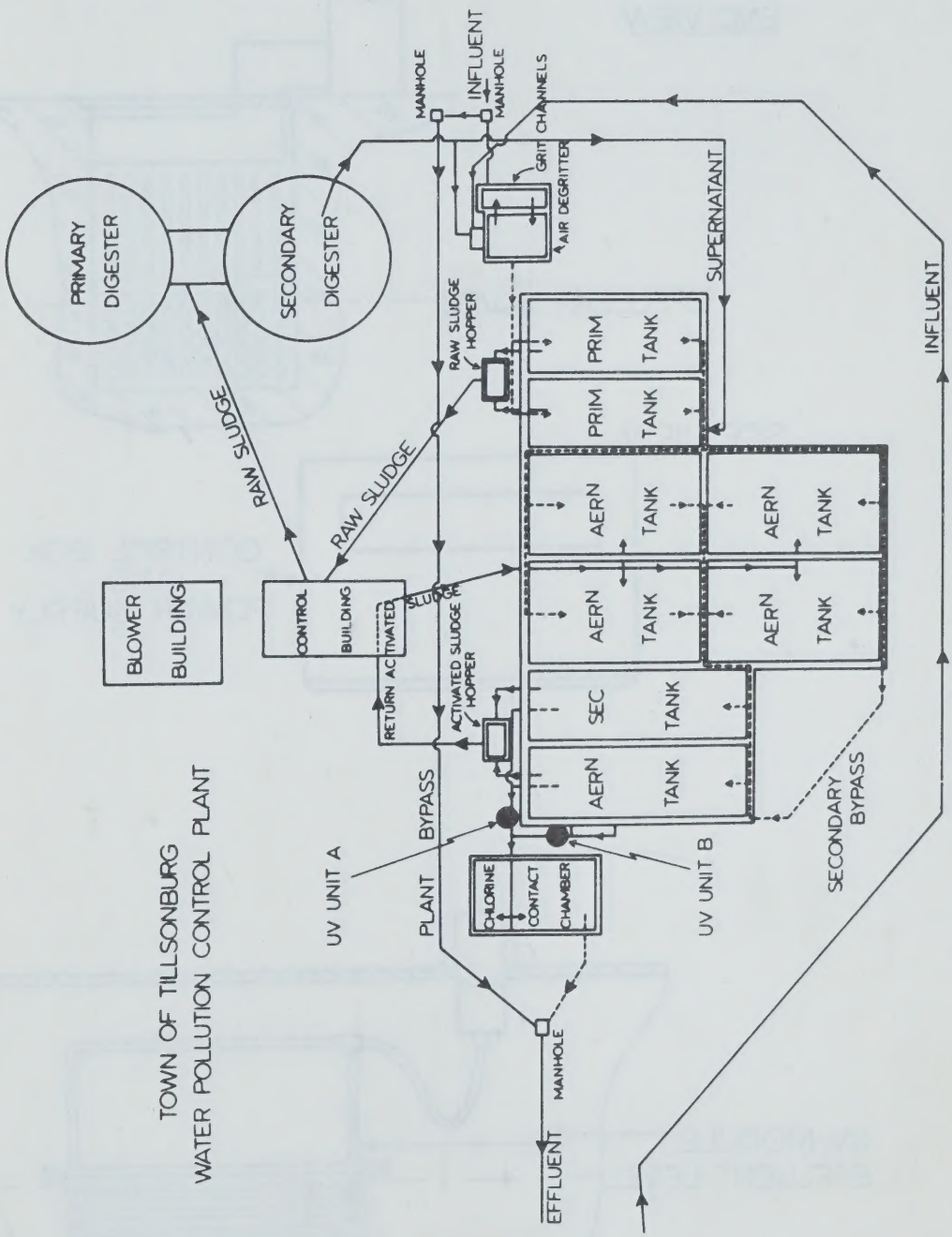


Figure 1. Design of the water pollution control plant.

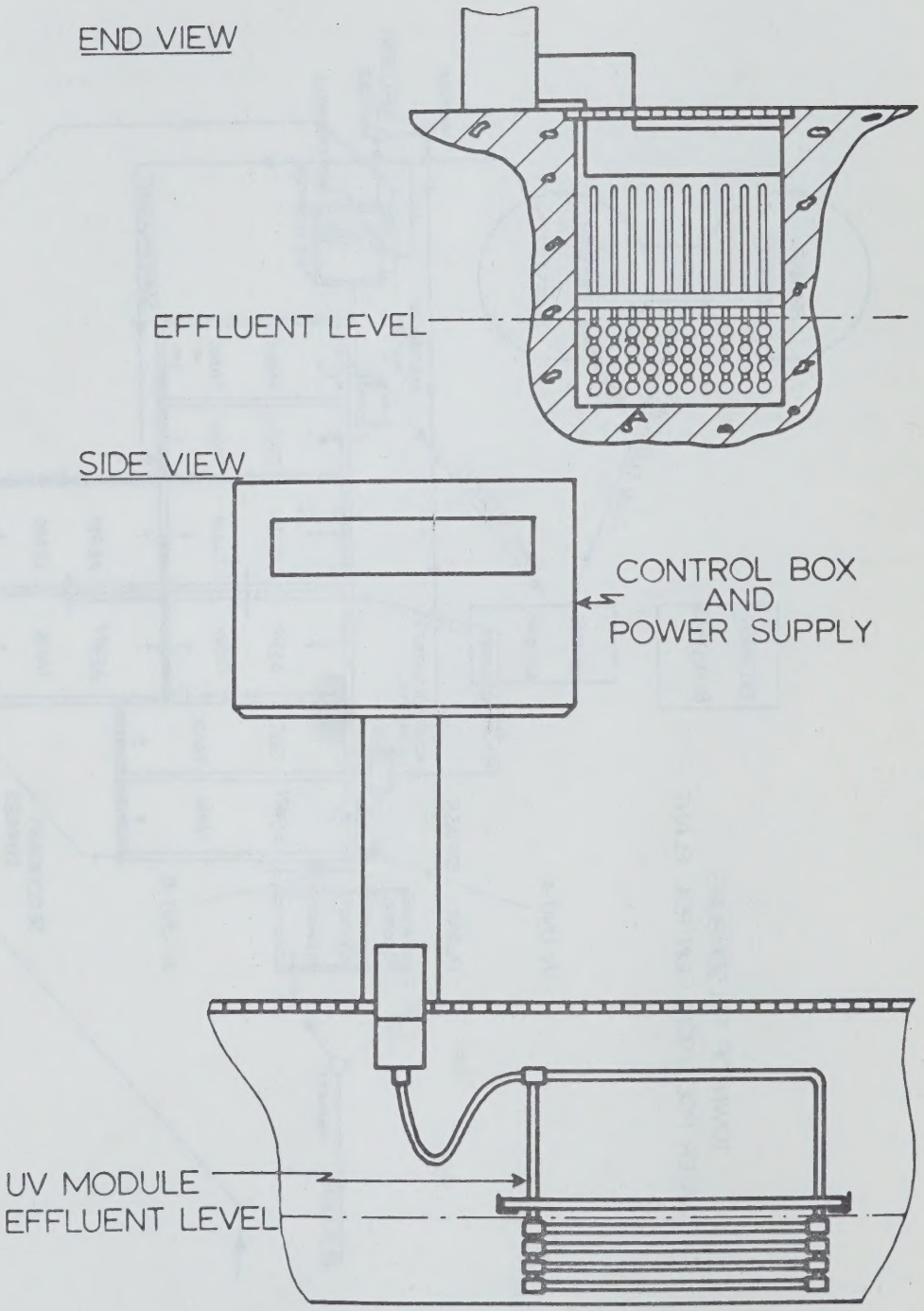


Figure 2. Schematic of the ultraviolet disinfection unit in the sewage treatment plant effluent channels.

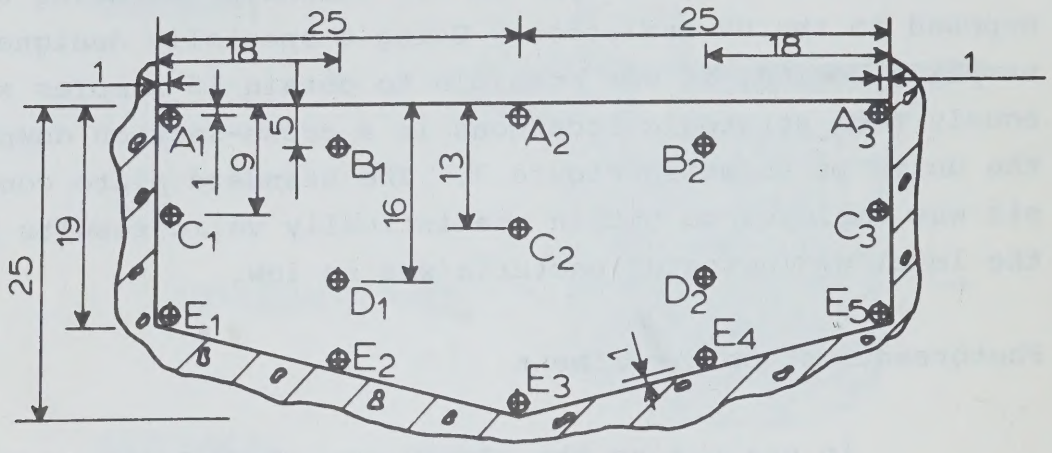
Uniform Exposure Experiment

A cross-sectional sampling of the effluents was conducted immediately downstream of Units A and B to determine whether all of the effluent in the channels was being uniformly exposed to the UV radiation. Using a specially designed vacuum sampling device, it was possible to obtain 15 samples simultaneously from strategic locations in a cross-section downstream of the units as shown in Figure 3. The standard plate count analysis was employed to obtain statistically valid results because the level of indicator bacteria was so low.

Photoreactivation Experiment

In evaluating the efficiency of UV light as a disinfectant, it is essential to assess the degree of recovery of bacteria following irradiation. Photoreactivation[16,17] results in an increased survival of bacteria following exposure to sunlight and dark repair[17] involves the excision of damaged DNA in the bacterial cell in the absence of light. A study was conducted to examine the effects of these repair systems. One homogeneous effluent sample was obtained after Unit A and was used to fill a series of 500 ml clear round Pyrex bottles. One duplicate series was kept in the dark by covering the bottles with tin-foil and another identical group was exposed to direct sunlight. To assess the potential effect of the receiving stream on bacterial recovery another duplicate light and dark series was prepared by diluting 1 part effluent with 3 parts filter sterilized Big Otter Creek water. The diluted effluent samples were designated 25% light or dark. All of the bottles were placed just below the surface of the settling tanks in special racks to simulate actual conditions.

CROSS SECTION OF UV UNIT A



ALL DIMENSIONS ARE IN CENTIMETERS
SAMPLING POINTS ⊕

CROSS-SECTION OF UV UNIT B

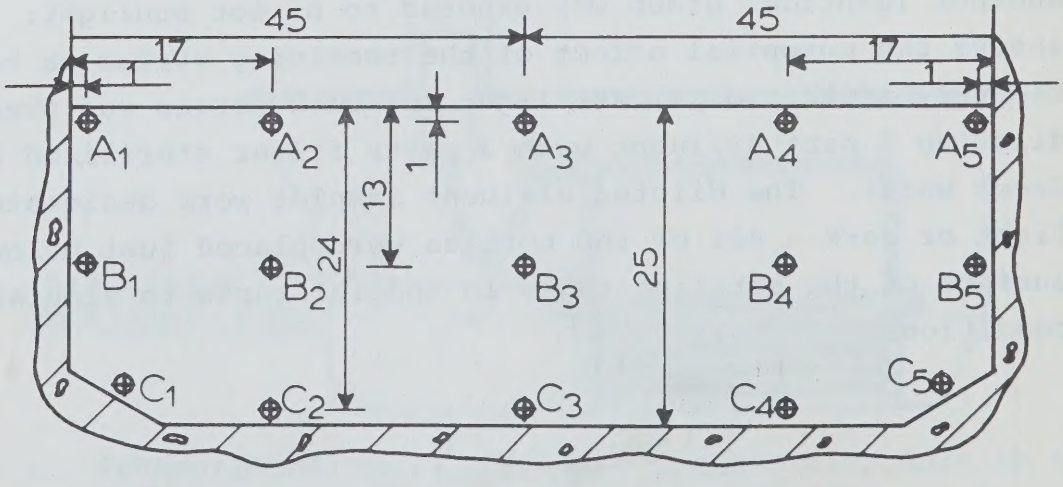


Figure 3. Cross-sections of the channels showing the 15 sampling points.

Routine Sampling

As of July 1982, chemical and bacteriological sampling was carried out on a routine basis on the influents, between the matrices and effluents following Units A and B and the chlorine contact chamber.

Unit B was turned off 18h before sampling to allow the chlorine contact chamber to equilibrate to normal. Samples of chlorinated and UV disinfected effluent were exposed to the sunlight entering the clarifiers. Chlorine residuals were determined on the chlorinated effluent samples by the DPD method. All samples were immediately returned to the laboratory where the chemical and bacteriological analyses were conducted according to the Ontario Ministry of the Environment's Handbook of Analytical Methods[18].

Fish Toxicity Assay

A fish-toxicity evaluation was conducted in the receiving stream to compare the effects of UV and chlorine disinfection of the effluent. Rainbow trout yearlings were exposed in cages above and below the outfall of the sewage treatment plant (Figure 4). A variety of chemical and microbiological parameters were measured in the plant and in the receiving stream during the exposures.

Assay of Bacteriophage to Escherichia coli C

The bacteriophage in the wastewater before and after UV and chlorine disinfection were enumerated to obtain an indication of the effect of disinfection on an organism which closely resembles enteric viruses which are much more difficult to enumerate. The MPN method of Kott[19] was modified by using the agar, broth and Escherichia coli C. host of Scott et al.[20] to enumerate the bacteriophage.

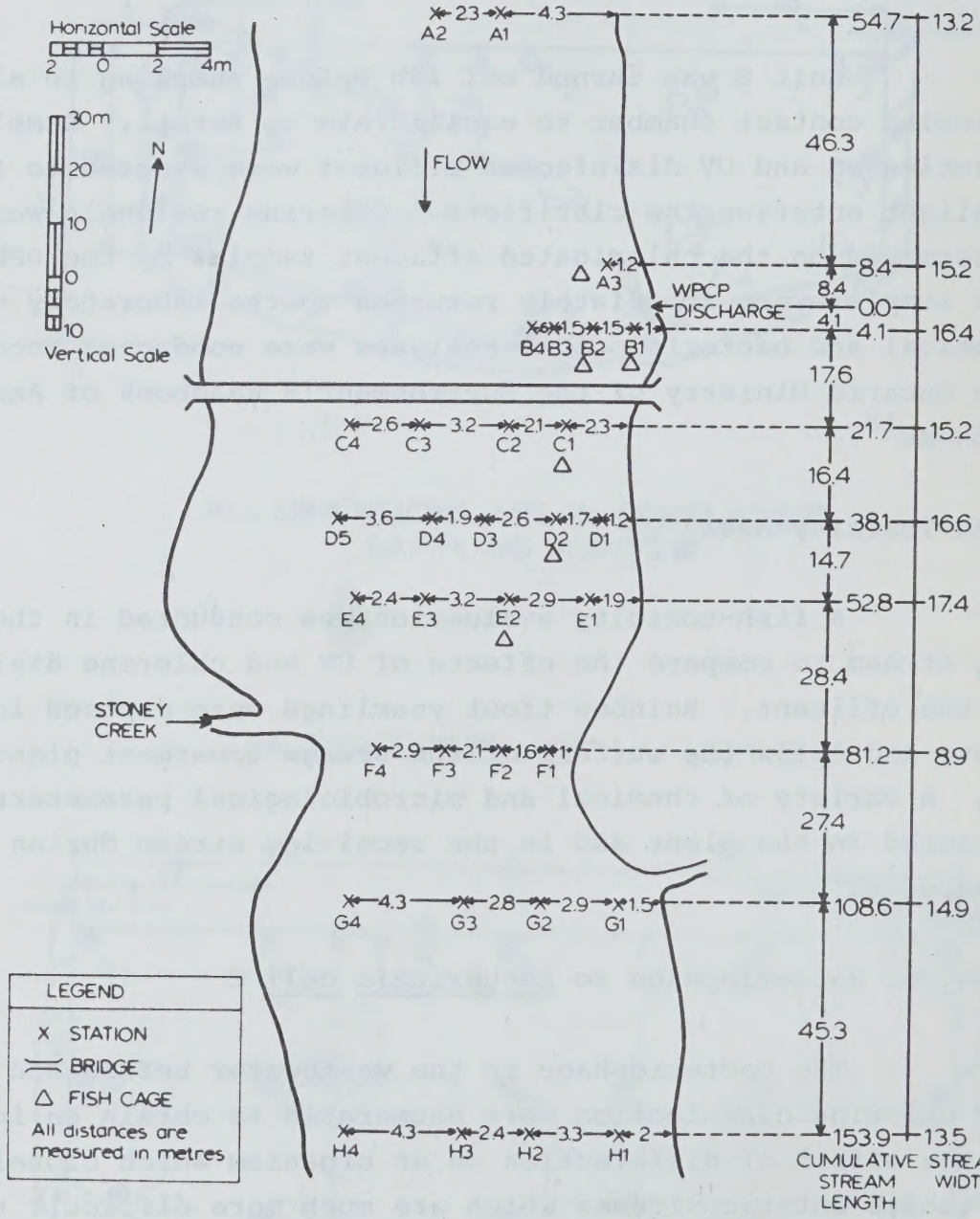


Figure 4. Location of fish cages in Big Otter Creek during the UV and chlorine disinfection toxicity tests.

Enumeration of Clostridium perfringens

The following procedure is used for the examination of C. perfringens, a spore-forming bacterium which is known for its disinfection resistance[21]. A sample of wastewater is filtered through a 0.45 um filter (Type GN-6, Gelman Sciences Inc.). The membrane is then inverted, placed on C. perfringens agar for 1 minute after which the membrane is removed and discarded. The C. perfringens agar plate is incubated anaerobically at 44.5°C for 24 h. Lactose fermenting and lecithinase positive colonies are considered to be typical of C. perfringens.

To differentiate the vegetative cells of C. perfringens from those in the spore form, samples were heated at 80°C for 10 min and then rapidly cooled to room temperature with cold water. The samples were then analyzed as described previously. Unheated controls were also included.

Pathogen Testing

The effect of UV disinfection on waterborne pathogens in wastewater is unknown. Therefore, the UV and chlorinated effluents were tested for the following pathogens.

Pseudomonas aeruginosa was enumerated by a membrane filter technique according to the Ontario Ministry of the Environment's Handbook of Analytical Methods[18].

The level of Yersinia enterocolitica was determined by the method of Schiemann[22].

The level of Campylobacter jejuni was estimated by the modified methods of Doyle and Roman[23] and Blaser et al[24].

Salmonella species were detected using either a qualitative method which was that of Palmateer and Koellner[25],

or a quantitative method. The quantitative analysis was a most probable number technique which included the filtration of approximately 10 l of sample using a sterile glass-fibre pre-filter and bacterial membrane filters of .45 um pore size. Nine samples of 3 separate 3-l, 300 ml and 30 ml aliquots were filtered after which each pre-filter and bacterial filter was placed in separate pre-enrichment broth labelled appropriately as to aliquot volume and source. At this point, further analysis is identical to that described above.

Cost Comparison

A cost comparison was prepared between the capital and operating costs of chlorination, dechlorination, chlorination-dechlorination, ozonation and UV disinfection by using the Innovative and Alternative Technology Assessment Manual of the EPA[26], the information from the UV disinfection project and data from Trojan Technologies Inc., London, Canada which manufactured the UV disinfection system. The costs in the Assessment Manual were updated to the 3rd quarter of 1982 by using the appropriate Engineering News Record Indexes. The amortization time is taken as 15 years and the interest rate is 12% semi-annually. The costing figures are reported as ratios to simplify the comparison between the various modes of disinfection.

RESULTS AND DISCUSSION

Uniform Exposure Experiment

As shown in Table 1, the geometric means of the standard plate count bacteria after 3 sets of samplings in both channels indicate an absence of short-circuiting of bacteria through the UV Units. This data showed that sampling at any location downstream of the UV Units would be representative of the degree of bacterial reduction.

Table 1. Results of the standard plate count of the cross-section sampling of Units A and B.

Unit A					Unit B				
Location	Bacteria per ml Sampling				Location	Bacteria per ml Sampling			
	1	2	3	GM*		1	2	3	GM*
A1	6	4	16	7	A1	2	11	3	4
A2	6	4	4	5	A2	2	2	1	2
A3	5	4	35	9	A3	2	3	0	1
B1	5	3	3	4	A4	3	2	1	2
B2	6	3	4	4	A5	3	8	1	3
C1	4	3	2	3	B1	1	4	0	1
C2	4	5	2	3	B2	2	1	1	1
C3	6	8	3	5	B3	2	2	1	2
D1	12	4	3	5	B4	2	1	0	1
D2	5	3	3	4	B5	2	137	1	6
E1	6	6	3	5	C1	3	1	1	1
E2	5	5	5	5	C2	3	3	6	4
E3	6	13	6	8	C3	2	3	4	3
E4	6	6	4	5	C4	1	2	1	1
E5	3	5	4	4	C5	3	12	3	5
Influent	2500	5700	2800	3417	Influent	2400	3400	3300	2997

*GM - Geometric Mean

Photoreactivation Experimentation

The data in Figure 5 shows the means of four separate photoreactivation experiments. Total coliform and fecal coliform results indicate a maximum increase of 1 logarithm whereas the fecal Streptococci failed to photoreactivate after 6h exposure to sunlight. A dark repair mechanism may have existed but it was undetectable in this data. Mixing the irradiated effluent with sterile Big Otter Creek water did not enhance the repair mechanisms after 6h. Conversely, any potential toxicity of the irradiated effluent to the indicator bacteria was not detectable in the samples diluted with creek water. Although the results were somewhat variable, a 3h exposure appeared to be optimal for the routine sampling study. A full scale study by Scheible and Bassell[15] showed that a 1h exposure was adequate for optimal photoreactivation. Factors such as disinfection efficiency and the specific nature of the wastewater being treated may account for the difference.

Routine Monitoring

The results of the bacteriological analysis of samples taken before, in between the two sets of UV modules in each unit and after UV Units A and B are shown in Tables 2 and 3. As can be seen in these tables, the geometric mean and the range of the total and fecal coliforms in the effluent following UV disinfection and subsequent photoreactivation are well below the suggested Ontario Ministry of the Environment Guidelines of 2500 total and 200 fecal coliforms per 100 ml of effluent. In between the two sets of UV modules of Units A and B the geometric mean of the total and fecal coliform numbers is below the suggested guidelines before and after phototreactivation but the range of the coliform numbers exceeds the limit. Therefore, both sets of UV modules are required if the coliform numbers are to be kept below the guidelines at all times. The average log increase in the total coliform numbers after 3 h photoreactivation was 0.99

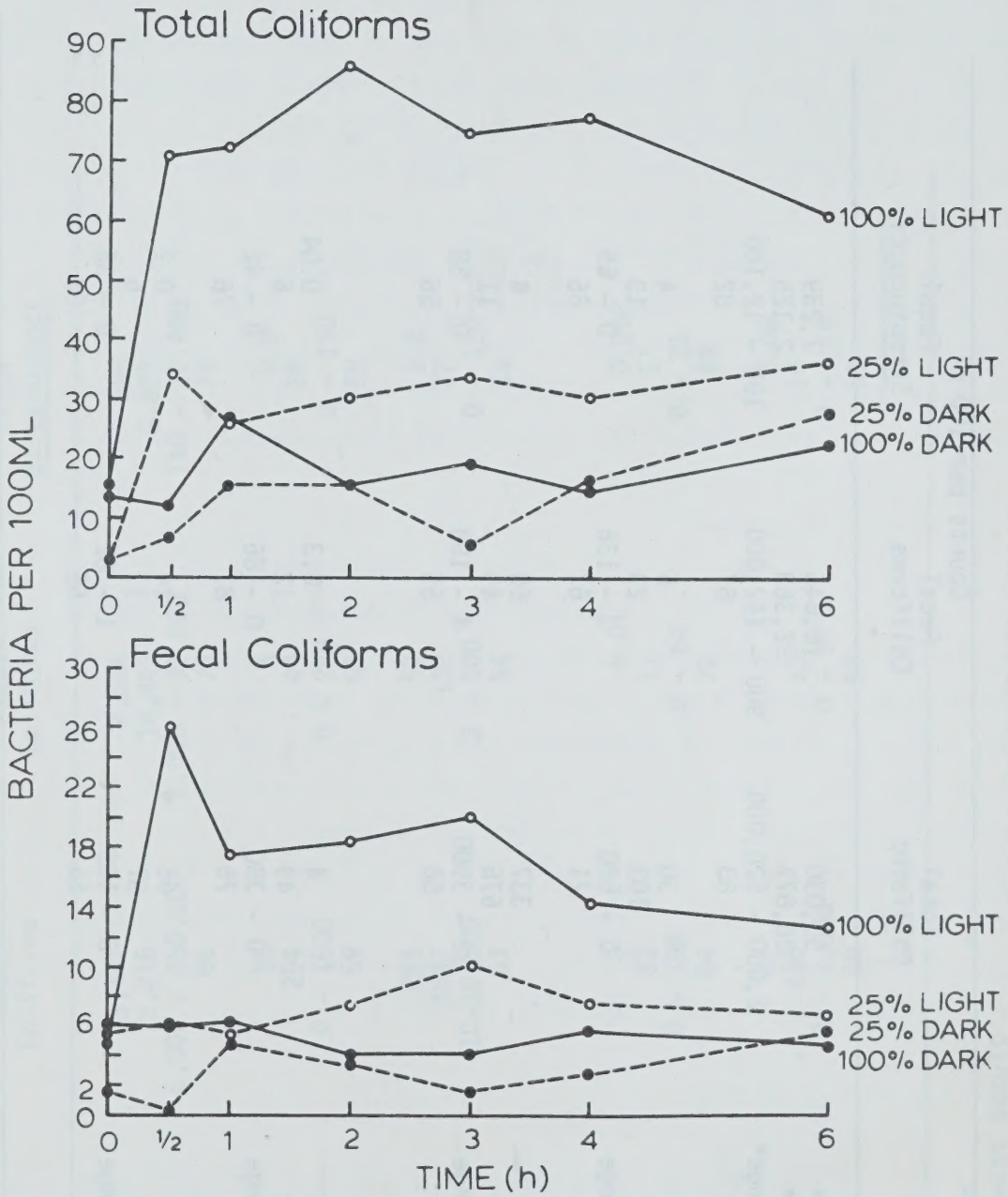


Figure 5. Results of exposing total and fecal coliform bacteria in full strength (100%) and diluted (25%) effluent to sunlight and dark controls following UV treatment from Unit A.

Table 2. Results of the routine bacteriological analysis of the wastewater before, in between the two sets of UV modules and after UV Unit A.

Sample Source		Counts per 100 ml Total Coliforms	Fecal Coliforms	Fecal Streptococci
Influent to Unit A	GM*	53,030	15,645	1,239
	SD*	86,877	22,363	2,125
	Range*	2,000 - 520,000	900 - 142,000	100 - 12,100
	N*	83	87	82
Effluent Between the Sets of UV Modules	GM	30	5	4
	SD	103	23	13
	Range	0 - 560	0 - 136	0 - 55
	N	71	67	66
Effluent Between the Set of UV Modules after 3 h Photo-reactivation	GM	337	50	4
	SD	676	49	11
	Range	32 - 3900	4 - 188	0 - 38
	N	56	55	56
Effluent from Unit A	GM	4	0.3	0.04
	SD	49	12	6
	Range	0 - 380	0 - 86	0 - 42
	N	75	81	76
Effluent from Unit A After 3h Photo-reactivation	GM	66	14	0.2
	SD	87	17	6
	Range	8 - 420	1 - 82	0 - 36
	N	65	65	62

*GM - Geometric Mean
SD - Standard Deviation
Range - Highest and Lowest Result
N - Number of Samples

Table 3. Results of the routine bacteriological analysis of the wastewater before, in between the two sets of UV modules and after UV Unit B.

Sample Source		Counts per 100 ml Total Coliforms	Fecal Coliforms	Fecal Streptococci
Influent to Unit B	GM*	39,447	7,636	598
	SD*	72,816	16,447	2,227
	Range*	6,000 - 490,000	1,000 - 92,000	140 - 17,400
	N*	66	73	70
Effluent Between the Sets of UV Modules	GM	35	6	0.9
	SD	294	49	28
	Range	0 - 1500	0 - 288	0 - 150
	N	59	61	59
Effluent Between the Sets of UV Modules after 3 hr Photo-reactivation	GM	291	57	1.2
	SD	1637	132	27
	Range	10-10,300	3 - 600	0 - 150
	N	53	54	54
Effluent from Unit B	GM	4	0.01	0.002
	SD	33	11	5
	Range	0 - 198	0 - 64	0 - 25
	N	64	72	69
Effluent from Unit B After 3h Photo-reactivation	GM	31	7	0.05
	SD	138	15	16
	Range	3 - 1,000	0 - 57	0 - 119
	N	58	59	58

*GM - Geometric Mean
SD - Standard Deviation
Range - Highest and Lowest Result
N - Number of Samples

and 1.08 respectively in between the 2 sets of UV modules in each unit and after the UV units. This is in agreement with the results of Scheible and Bassell[15] but not Bohm et al[28] who found an increase of 1.4 to 1.7 orders. The UV energy fluence had no effect on the degree of photoreactivation as it remained almost identical in between the UV modules of each unit and after the units. The fecal Streptococci were reduced substantially by the UV units and remained low as this group failed to photoreactivate as was reported by others[14]. Table 4 shows that the indicator bacterial levels in the chlorinated effluent are similar to the levels found in the UV irradiated effluent following photoreactivation. The chemical analyses (Table 5) conducted on the effluents at the time of bacterial analyses indicated a good quality effluent, which was free of any toxic chemicals which would have complicated any analyses during this study.

Fish Toxicity Assay

Results of chemical analyses conducted on the effluent on site during the toxicity assay are shown in Table 6. During the chlorination study, the effective time to death for 50% of the test fish was 7h from the test initiation at all downstream stations and complete mortality occurred within 24h. During an initial exposure to UV disinfected effluent, a few fish mortalities were observed (maximum of 14% at any one site). It was attributed to primary sewage which bypassed the plant treatment system during a storm event. A repeat UV disinfection test was non-lethal for a 48h exposure period.

Bacteriophage Assay

The results in Table 7 show that Units A and B killed 99.97 and 99.98% respectively of the bacteriophages whereas chlorination killed 95.1%. This indicates that UV irradiation may be more effective at reducing the level of enteric viruses in wastewater.

Table 4. Results of the routine bacteriological analysis of the effluent before and after chlorine disinfection.

Sample Source		Counts per 100 ml Total Coliforms	Fecal Coliforms	Fecal Streptococci
Influent to the Chlorine Contact Chamber	GM*	40,827	7,203	578
	SD*	78,928	12,600	2,203
	Range*	6,000 - 490,000	84 - 81,000	140 - 17,400
	N*	55	60	60
Effluent from the Chlorine Contact Chamber	GM	101	0.4	14
	SD	4,482	205	56
	Range	0 - 21,800	0 - 1,500	0 - 286
	N	56	61	61
Effluent from the Chlorine Contact Chamber after 3h Exposure to Sunlight	GM	98	4	0.2
	SD	959	62	56
	Range	10 - 6,700	0 - 310	0 - 340
	N	54	55	55

*GM - Geometric Mean
SD - Standard Deviation
Range - Highest and Lowest Result
N - Number of Samples

Table 5. Means of the measured chemical parameters from July 1982 to September 1983

Parameters	Influent to Unit A	Influent to Unit B	Effluent from Chlorine Contact Chamber
Flow Rate 10^3 x m^3/d	3.7	2.9	
Chlorine residual mg/l			0.4
%T*, 254 nm, 1 cm	77.9	77.5	77.9
%T, 365 nm, 1 cm	94.6	94.3	94.5
Dissolved organic carbon mg/l	4.2	4.3	4.5
Apparent colour in Hazen Units	18.4	17.9	15.4
Turbidity in Formazin Units	1.9	1.8	2.2
pH	7.1	7.1	7.1
Conductivity in umho/cm^3 at 25°C	1011.3	986.8	997.6
Iron as Fe mg/l	0.037	0.027	0.032
Nitrogen mg/l			
(1) Free ammonia	0.4	0.6	0.8
(2) Total Kjeldahl	1.2	1.4	1.9
(3) Nitrite	0.3	0.3	0.3
(4) Nitrate	18.3	17.1	17.1
Phosphorus as P			
(1) Total mg/l	0.62	0.57	0.57
(2) Dissolved reactive mg/l	0.21	0.19	0.19
BOD mg/l	2.8	2.4	2.1
Suspended solids mg/l	6.1	5.3	5.6
Dissolved solids mg/l	766.5	653.8	648.2
COD	14.6	14.6	14.3

*%T - Percent Transmission

Table 6. Chemical parameters measured on site during the fish toxicity study.

	UV Run 1	UV Run 2	Chlorine Run
Chlorine Residuals			
(1) Total Chlorine Residual ug/l	0	0	178 - 1,510
(2) Monchlaromine ug/l	0	0	92 - 1,080
(3) Dichloramine ug/l	0	0	128 - 600
Nitrogen mg/l			
(1) Free ammonia	0.048 - 4.500	0.002 - 1.02	0.020 - 0.950
(2) Total Kjeldahl	0.69 - 1.63	0.63 - 1.30	0.63 - 2.65
pH	7.20 - 8.20	7.24 - 7.47	7.09 - 7.48
Conductivity umho/cm^3 at 25°C	463 - 1,039	940 - 1,170	825 - 1,130
Phenols ug/l	*LO.2 - 8.8	*N.D.	1.2 - 2.4

*N.D. - Not Done
L - Less Than

Table 7. Results of the MPN Method of counting bacteriophages to *E. coli* C before and after UV and chlorine disinfection.

Sample Source	Bacteriophage counts per 100 ml Geometric Mean	Standard Deviation	Number of Samples
Influent to Unit A	3,530	12,568	12
Effluent from Unit A	0.95	4.62	12
Influent to Unit B and the Chlorine Contact Chamber	6,233	40,898	12
Effluent from Unit B	1.4	3.4	11
Effluent from the Chlorine Contact Chamber	304	3,205	11

Table 8. Results of the routine enumeration of C. perfringens

Sample Source	Counts per 100 ml Geometric Mean	Standard Deviation	Range	Number of Samples
Influent to Unit A	1,854	1,296	100 - 9,000	71
Effluent Between the Two Sets of UV Modules in Unit A	618	958	0 - 3,900	56
Effluent Between the Two Sets of UV Modules in Unit A after 3h Photoreactivation	801	936	100 - 3,600	51
Effluent from Unit A	358	626	16 - 2,600	66
Effluent from Unit A After 3h Photoreactivation	323	349	10 - 1,900	56
Influent to Unit B	1,625	1,174	248 - 5,900	62
Effluent Between the Two Sets of UV Modules in Unit B	402	890	8 - 3,700	51
Effluent Between the Two Sets of UV Modules in Unit B after 3h Photoreactivation	249	803	0 - 3,100	51
Effluent from Unit B	27	205	0 - 830	61
Effluent from Unit B after 3h Photoreactivation	81	204	2 - 850	54
Influent to the Chlorine Contact Chamber	1,683	1,056	500 - 5,400	54
Effluent from the Chlorine Contact Chamber	1,292	1,068	76 - 4,500	55
Effluent from the Chlorine Contact Chamber after 3h Exposure to Sunlight	814	1,147	0 - 5,300	50

Enumeration of C. perfringens

The routine results from Table 8 show that Units A and B killed approximately 1.8 times as many C. perfringens as chlorination. Based on the differentiation of vegetative cells and spores, neither UV or chlorine appeared to affect the spores.

Pathogen Testing

As shown in Table 9, UV and chlorine disinfection were both equally effective in destroying Pseudomonas aeruginosa.

In two preliminary runs, Yersinia enterocolitica was reduced equally by both modes of disinfection. However, due to the sensitivity of the bacterium to warm waters, further analysis will commence when the temperature of the effluent is below 10°C[27].

Using the most current isolation methods, Campylobacter jejuni was not detected in the undisinfected wastewater.

The quantitative analysis revealed that UV and chlorine were equally efficient at reducing the Salmonella population. The qualitative method showed that Salmonella sp were present in the UV irradiated effluent but not in the chlorinated effluent as detected by a Moore swab suspended in the effluents for 24-96 h. The absence of Salmonella in the chlorinated effluent may be due to the residual of chlorine which continuously bathed the swabs.

Table 9. Results of the analysis for Pseudomonas aeruginosa in the effluents before and after UV and chlorine disinfection

Sample Source	Counts per 100 ml Geometric Mean	Standard Deviation	Range	Number of Samples
Influent to Unit A	151	321	24 - 1500	26
Effluent from Unit A	1	0.3	*L4 - 0	25
Effluent from Unit A after 3h Photoreactivation	1.1	0.75	3 - 0	20
Influent to Unit B and the Chlorine Contact Chamber	153	413	16 - 1500	23
Effluent from Unit B	1.0	0.46	2 - 0	22
Effluent from Unit B after 3h Photoreactivation	1.0	0.4	L4 - 0	19
Effluent from the Chlorine Contact Chamber	1.4	21	0 - 101	22
Effluent from the Chlorine Contact Chamber after 3h Exposure to Sunlight	1.3	7.3	32 - 0	19

*L - Less Than

Cost Comparison

The ratios in Table 10 show that UV disinfection can be a cost effective alternative to other modes of disinfection. UV lamp replacement and power usage make up the majority of the UV operating costs when the amortization costs are excluded. The lamp replacement time has been taken as 18 months because in this study the UV lamps have operated consistently for 18 months. Ozonation is not cost competitive with UV irradiation because ozonation has high installation and operating costs.

Maintenance Observations

Although current results have remained consistently satisfactory, the UV lamps have been cleaned once. During 18 months of total operation, no masses of algae have been observed to build up on the units because the UV lamps are parallel to the flow. Five UV lamps have been replaced during the 18 months.

CONCLUSIONS

1. These results show that UV disinfection of treated wastewater is a viable alternative to chlorination if the UV unit is designed specifically for this purpose.

2. This study demonstrated that UV devices can be designed which require minimal maintenance and very little on-site modifications.

3. UV irradiation of wastewater can reduce the bacterial concentrations to safe levels without introducing a toxicant to fish in the receiving waters.

Table 10. Comparision of capital and operating costs for chlorination, dechlorination, chlorination-dechlorination, ozonation and ultraviolet disinfection in the United States

	Capital Cost Ratios Plant Size (10^3 m^3/d)			
Disinfection Mode	3.78	18.9	37.8	378
Chlorination	2.01	1.76	1.45	0.86
Dechlorination	0.68	0.48	0.36	0.14
Chlorination-Dechloration	2.69	2.24	1.81	1.00
Ozonation by air	9.98	7.46	7.27	6.96
UV (Trojan UV2000 System)	1	1	1	1
	Operation Cost Ratios			
Chlorination	2.77	2.46	2.11	1.47
Dechlorination	1.19	0.90	0.72	0.41
Chlorination-Dechlorination	3.96	3.36	2.84	1.88
Ozonation by air	8.67	6.87	6.73	7.05
UV (Trojan UV2000 System)	1	1	1	1

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FIELD MEASUREMENT OF INFILTRATION THROUGH LANDFILL COVERS

BY

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ABSTRACT:

The usual source of landfill leachate is related to the infiltration of precipitiation through the soil cover and then refuse. The prediction of infiltration through landfill surface covers is often qualitative and is poorly documented. Minimal data are available to assess the effectiveness, reliability and design of current and past practices relative to leachate production. A two phased study, of which this is the first, is to be carried out over a three year period. The objectives of Phase I reported here are to design a suitable lysimeter applicable to a variety of landfill environments that will provide economical repeatable and accurate measurements. In Phase 2 a number of variables and settings will be documented. A literature search and review was the initial task undertaken and provided the basis for design. Installations were constructed at the Britannia Road Landfill in Mississauga in the Region of Peel. This site is an engineered facility with cells constructed and

based in low permeability clay subsoils. The landfill is a containment site with provisions for leachate collection and off-site treatment. The final soil cover is a compacted silty clay. Two types of lysimeters are reported upon, one being of a sophisticated data accumulation type including a storage well and an automatic data recording mode while the other is a simple sheet lysimeter. The storage well lysimeter has met the objectives.

The paper presents details of both designs along with installation and operation aspects and problems. Data accumulated since March, 1983 are presented along with preliminary analysis and recommendations.

INTRODUCTION:

One of the major controlling parameters required to properly design solid waste disposal sites with regard to leachate collection aspects and impact predictions, is of course the rate of leachate generation. Where landfilling is carried out above the ground water table, leachate generation is directly proportional to the amount of infiltration of precipitation which occurs through the landfill cover material. Studies have been carried out in the United States, namely for the Environmental Protection Agency, dealing with the design and assessment of landfill covers. Present design practice utilizes both empirical values and agricultural drainage equations together with climatic factors, in order to predict likely infiltration volumes

and thus leachate generation rates. No hard data are available for Ontario landfills except for a few studies that are of a general form. In the situations where leachate volumes have actually been measured, the rate of generation of leachate is invariably greater than that originally predicted.

The overall goal of the present project is to collect field measurements of the actual infiltration through various types of final cover materials, located in varying physical settings. The project is designed in two phases and is intended to be completed in approximately three years.

This paper reports on Phase 1 of the project, the main objective of which is to design and construct a field lysimeter installation that will provide economical, accurate and reproduceable measurements of infiltration at an existing landfill site.

LITERATURE REVIEW:

The first task of the project was to complete a literature review of past practices for measurement of infiltration in general and lysimeter installations in particular. A list of references is provided at the end of the paper. Most references encountered were concerned with the measurement or prediction of surface drainage or infiltration from the standpoint of either crop irrigation

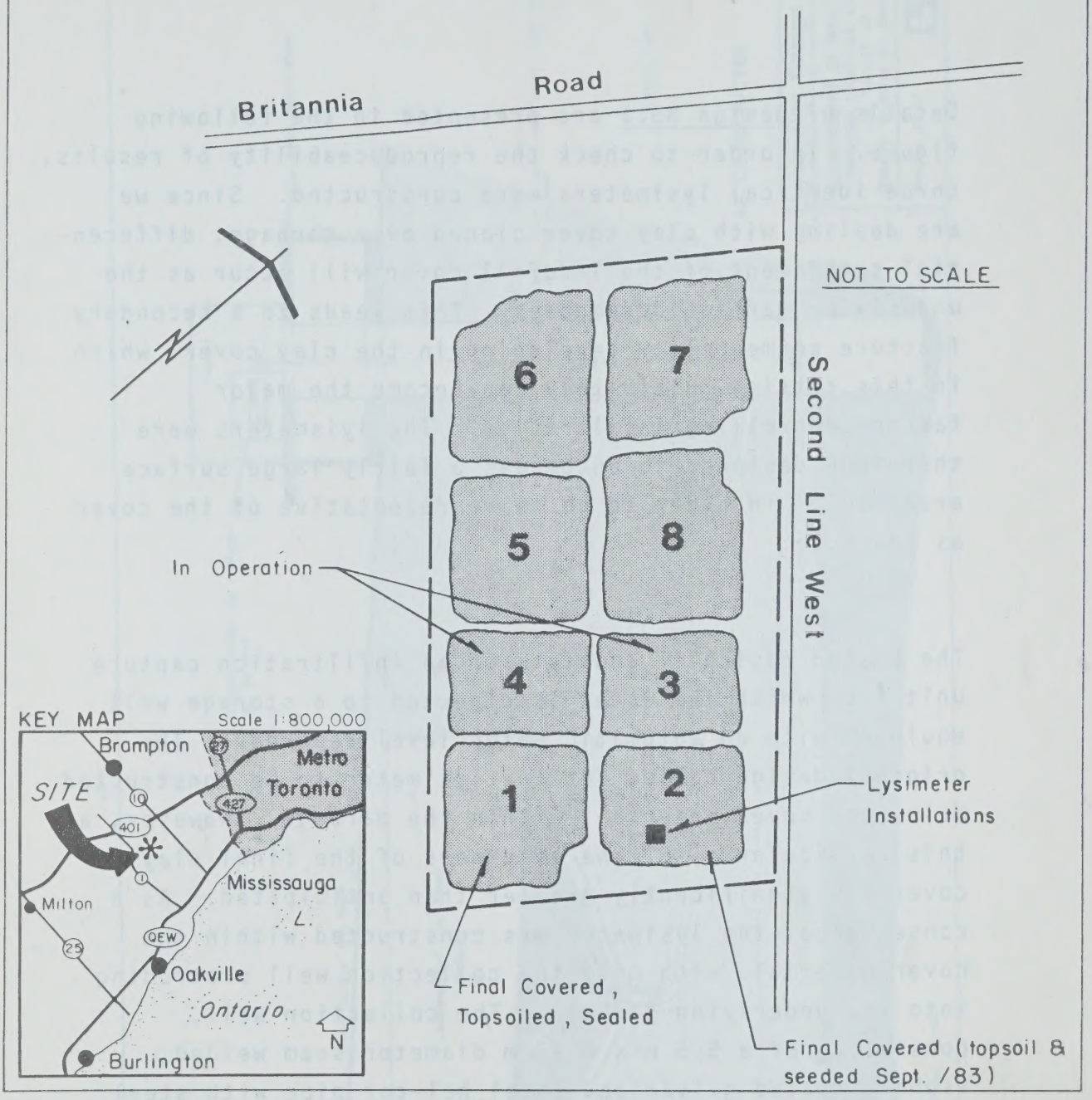
or ground water recharge. No references to the use of lysimeters for measurement of infiltration through landfill covers were found.

Site Location:

The site chosen for the installation of the lysimeters was the Central Britannia Road Landfill Site, Mississauga, located in the Region of Peel. This site is an engineered facility with cells constructed and bottomed in low permeability Halton clay till subsoils. The site layout is shown on the following figure. The landfill is a leachate containment site with provisions for full leachate collection by means of underdrains and off-site treatment. The final soil cover is a well compacted silty clay till, which is topsoiled and seeded.

Lysimeter Design:

Originally, only one lysimeter design was proposed, of a relatively sophisticated data accumulation type, that included a storage well and automatic data recording mode. Subsequent to undertaking the project, it was suggested by MOE staff that a much simpler type of sheet lysimeter should also be constructed and the results compared to those from the more sophisticated design.



BRITANNIA ROAD LANDFILL CELL LAYOUT

Fig. 1

MEASUREMENT OF INFILTRATION

Legend

Landfill Boundary

Approximate Outline Of Cell

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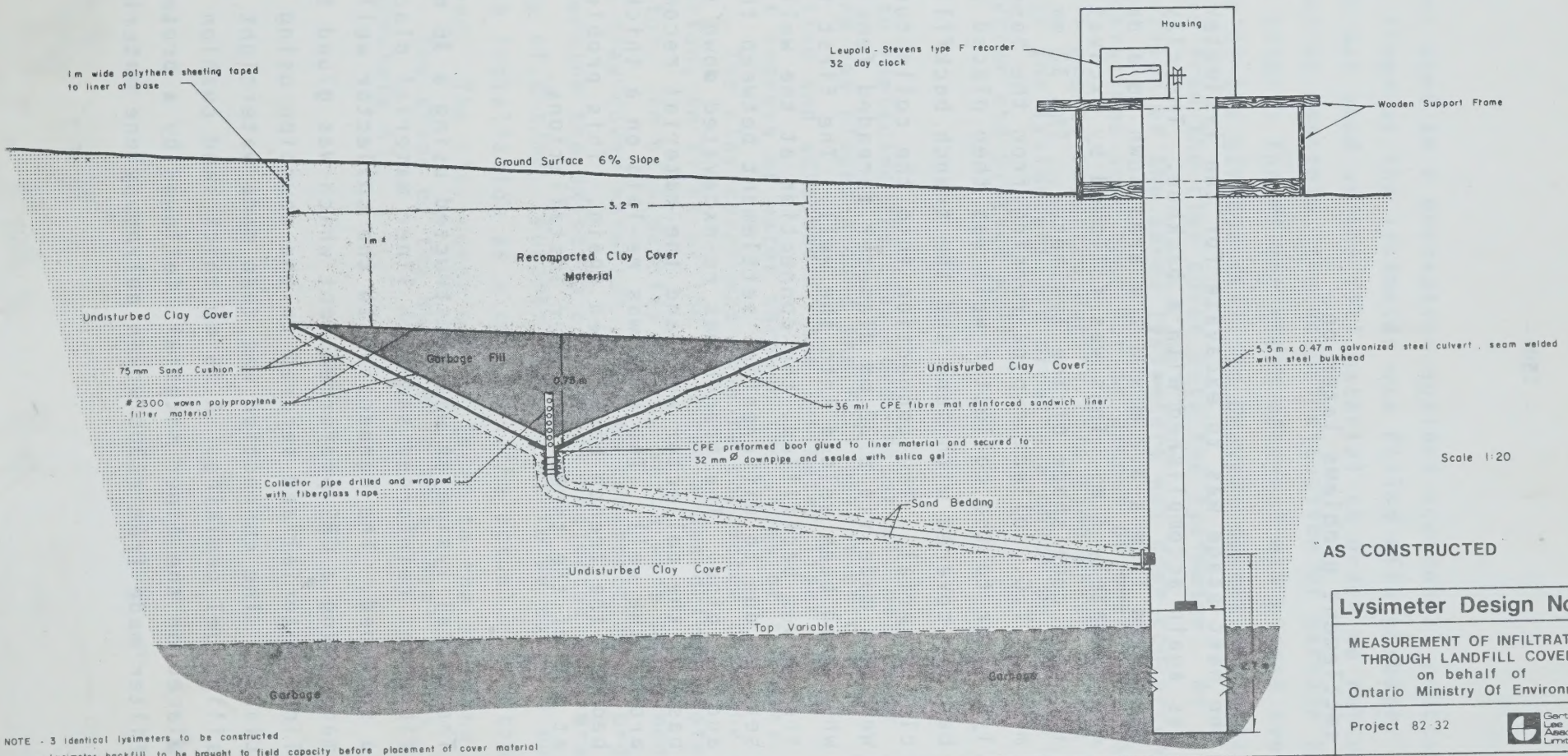
Details of design No.1 are presented in the following figure. In order to check the reproduceability of results, three identical lysimeters were constructed. Since we are dealing with clay cover placed over garbage, differential settlement of the landfill cover will occur as the underlying garbage decomposes. This leads to a secondary fracture permeability developing in the clay cover, which in this setting, ultimately can become the major factor controlling infiltration. The lyismeters were therefore designed to encompass a fairly large surface area, 10 m^2 in order to be as representative of the cover as possible.

The design basically consists of an infiltration capture unit from which the water is directed to a storage well equipped with an automatic water level recorder. The original design called for the lysimeter to be constructed below the cover material, within the garbage. However, at this particular site, the thickness of the final clay cover was significantly greater than anticipated. As a consequence, the lysimeter was constructed within the cover material, with only the collection well protruding into the underlying garbage. The collection well, consisting of a 5.5 m x 0.47 m diameter seam welded, asphalt coated galvanized steel hel-cor pipe with steel bulkhead, was installed first in late October 1982. The pipe was installed by means of a backhoe excavator because it was not possible to penetrate the garbage with a large diameter stirling auger drill rig, as was originally planned. The size of the excavation required in order to install the collection well and subsequent backfilling, did create some



NOTE - 3 identical lysimeters to be constructed
- Lysimeter backfill to be brought to field capacity before placement of cover material

Lysimeter Design No. 1

MEASUREMENT OF INFILTRATION THROUGH LANDFILL COVERS
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settlement problems later on.

The next stage was to excavate for the lysimeter, which was again accomplished with a backhoe. The clay cover was excavated from a 3.2 m x 3.2 m area down to a depth of approximately 1 m. This was followed by excavation of the inverted cone shape of the lysimeter. The 32 mm diameter P.V.C. collector pipe running from the base of the lysimeter to the collector well was then placed on a sand bed and covered with sand and the trench backfilled with clay material. The connection into the collector well was made via a 32 mm diameter threaded female stub which had been welded into the well. The first time the trench was backfilled, the connection at the well severed due to differential settlement between the well and the pipe. The trench was re-excavated down to the base of the collector well and the material recompacted around the well. The pipe was re-laid on a thicker sand bed and the trench backfilled again. This problem occurred at two of the three lysimeter installations.

The acutal lysimeter was constructed using a 36 mil CPE fibre mat reinforced sandwich liner material placed on a sand bed. The connection to the collector well was made using a CPE preformed boot which was glued to the liner material and secured to the downpipe using screw clamps. The connection was then made watertight using silica gel caulking. A 75 mm thick sand cushion was placed on the liner material, followed by a protective filter made from #2300 woven polypropylene material.

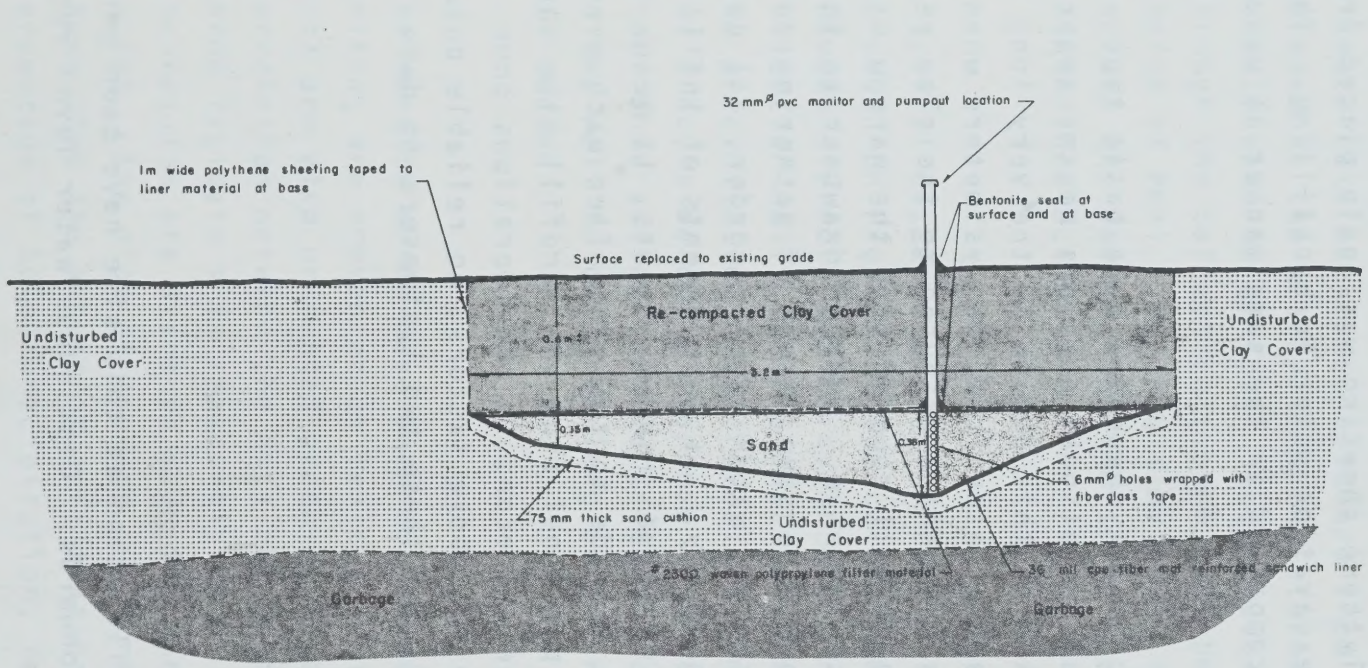
This was used as a precaution against accidental puncturing of the liner as the lysimeter was filled with garbage. Garbage was used as the fill material in order to reflect as close as possible, the actual situation of garbage beneath a clay cover. The filter cloth material was placed over the top of the garbage fill, to prevent downward migration of fines from the clay cover into the lysimeter. As construction of the three lysimeters was done concurrently, the excavations were open for in excess of one week. Several rainfall events occurred during this time which served to saturate the lysimeters and provide a check on the collection system and storage well.

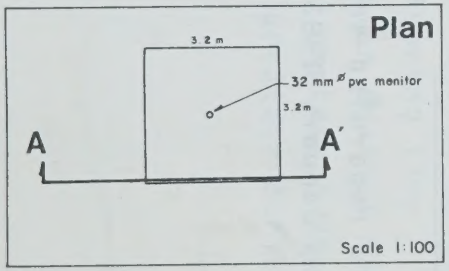
Prior to replacing the clay cover material, polythene sheeting was placed around the full height of the excavation and attached to the lysimeter liner to prevent ingress of water through the sides of the excavation. The clay cover was then re-compacted into the excavation, in lifts of 150 mm using a gas-driven plate compactor. In situ density tests, which were performed on the clay cover material prior to construction in order to determine the existing conditions indicated an average density of 2 t/m^3. Further testing is to be carried out at each installation as a check on the actual compaction achieved. The clay cover was replaced to the existing grade. The slope of the surface at this location was approximately 6%.

The final stage of the construction was to install the Leupold-Stevens type F continuous chart water level recorders. The recorder housings were built to MOE specifications and supported on a wooden framework which also served as a surface support system for the collector wells. The chart recorders, which are equipped with a battery operated, 32 day clock, have been operational since Mid-March 1983. Since it had been necessary to pump out water from the collection wells on several occasions, both during and after construction, it was assumed that the installations were at field capacity by the time the recorders were operative. Removal of water from the collection wells is accomplished by means of a contractors sump pump, equipped with a 6 m intake line. The ground surface at the installations remained without topsoil until the area was seeded in early September.

Details of lysimeter design No. 2 are presented in the following figure. The design consists of a simple sheet lysimeter equipped with a 32 mm diameter PVC monitoring and pump out pipe. There is no storage well nor automatic water level recording mode with this design.

Two lysimeters again 3.2 m x 3.2 m surface area were constructed, one using a sand fill and one using a garbage fill. The design consists of a 36 mil CPE fiber mat reinforced sandwich liner placed on a sand cushion. The lysimeter is approximately 0.4 m deep at the lowest point beneath the clay cover. #2300 woven polypropylene filter





"AS CONSTRUCTED"

SCALE 1:20

NOTE : 2 lysimeters to be constructed :
1 to be backfilled with garbage
2 to be backfilled with sand

Surface water run-off collection only at lysimeter 1

Lysimeter backfill to be brought to field capacity before placement of cover

Lysimeter Design No. 2

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material was again used to prevent movement of fines from the clay cover into the lysimeter. The thickness of clay cover above the lysimeter was reduced to approximately 0.6 m. Polythene sheeting was again placed around the sides of the excavation prior to backfilling. The clay cover was recompacted in the same manner as before.

Following construction of these two more basic type lysimeters, several operational problems became apparent. The major drawback with this design is the very long time required to remove the water from the lysimeters when they become full. This has been attributed to the slow response time of the lysimeter fill coupled with the narrow diameter of the monitoring pipe. Another drawback to this design is the labour intensive nature of gathering data. As there is no automatic water level recorder, all data must be obtained manually. If measurements of infiltration are required for individual ranfall events, it means that special trips must be made to the site after each event. Obviously, unless personnel from the landfill site can obtain the data, severe economic considerations come into play. Due to the above factors, no reliable data have been obtained from these two lysimeters to date.

Results To Date:

Data regarding infiltration at this site have been generated from January 1983 onwards. Continuous water level readings

have been obtained at the three sophisticated type lysimeters from mid-March onwards and all three installations appear to be functioning satisfactorily. Monthly records of climate data have been obtained from the weather station located at the nearby Toronto International Airport. Monthly records of total leachate volumes which pass through the collection system have been provided by the Region of Peel. It should be noted that these figures include the sewage from the scale house, site office and maintenance buildings.

A typical response curve for the lysimeter installations is shown on Figure 3. The curve shows a good correlation between a precipitation event and a response in the collection well. A more detailed look at the time of the precipitation event reveals a time lag of between 1 and 6 hours before a response is noted in the well. The other interesting feature is the tendency for a response in the well to continue long after the precipitation event has ceased.

The results which have been collected to the time of writing are presented on Table 1, which follows. These data are also presented graphically on Figure 4. The correlation between the data obtained so far from the three lysimeters is reasonably good, particularly when the results are averaged over the monitoring time period. The plot of average infiltration against time shows that the amount of infiltration, expressed as a percentage of total precipitation, is greatest during

TYPICAL MONITOR WELL RESPONSE CURVE
Lysimeter Installation #2 May 16 - June 15

Fig. 3

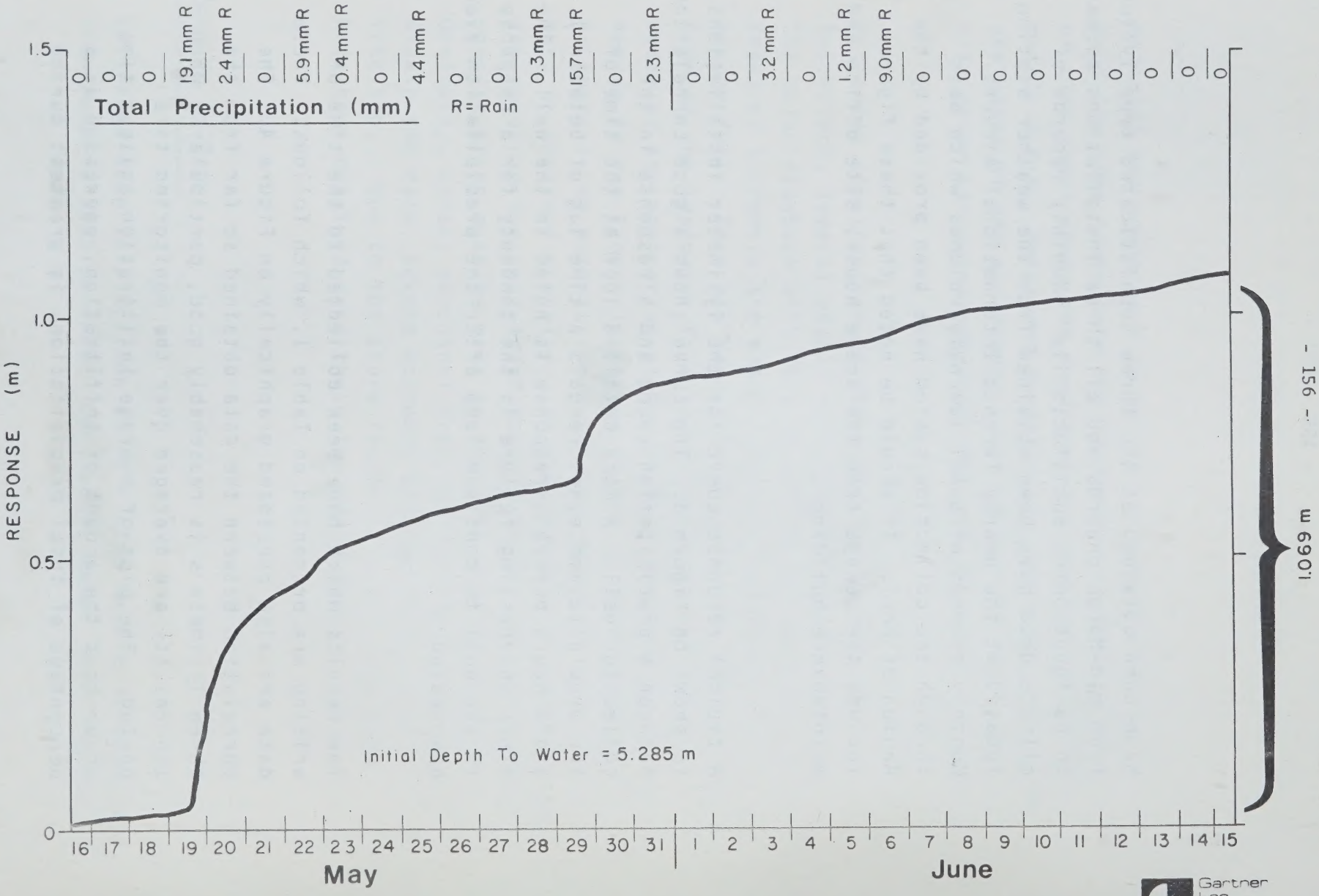

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Table 1: VALUES OF LYSIMETER INFILTRATION, MONTHLY PRECIPITATION AND TOTAL LEACHATE VOLUMES

Monitor	Total Precip. (mm Water)	LYSIMETER #1 INFILTRATION			LYSIMETER #2 INFILTRATION			LYSIMETER #3 INFILTRATION			LEACHATE COLLECTION SYSTEM
		VOLUME (LITRES)	EQUIV. DEPTH (mm)	AS % OF PRECIP.	VOLUME (LITRES)	EQUIV. DEPTH (mm)	AS % OF PRECIP.	VOLUME (LITRES)	EQUIV. DEPTH (mm)	AS % OF PRECIP.	TOTAL VOLUME PUMPED
											(cubic m)
Jan. - Mar.17	96	439	43.9	46	407.7	40.8	43	808.5	80.9	63	Jan. 4535
Mar.18 to Mar.31	55.7	513.5	51.4	92	449.5	45.0	81	21st on 157.3	15.7	66	Feb. 7617 Mar. 5756
Apr.	83.2	140.1	14.0	17	86.2	8.6	10	354.3	35.4	43	3723
May	99.5	163.9	16.4	17	208.9	20.9	21	121.6	12.2	12	4035
June	33.0	68.7	6.9	21	63.5	6.4	19	34.4	3.4	10	2377
July	18.3	35.1	3.5	19	3.5	0.35	2	7.3	0.7	4	2090
August	112.2	79.8	8.0	7	34.7	3.5	3	78.1	7.8	7	1750
Sept.											
Oct.											
Nov.											
Dec.											
TOTAL				Average 31%			Average 26%			Average 29	

FIGURE 4

MEASUREMENT OF INFILTRATION THROUGH LANDFILL COVER

ON BEHALF OF

ONTARIO MINISTRY OF THE ENVIRONMENT

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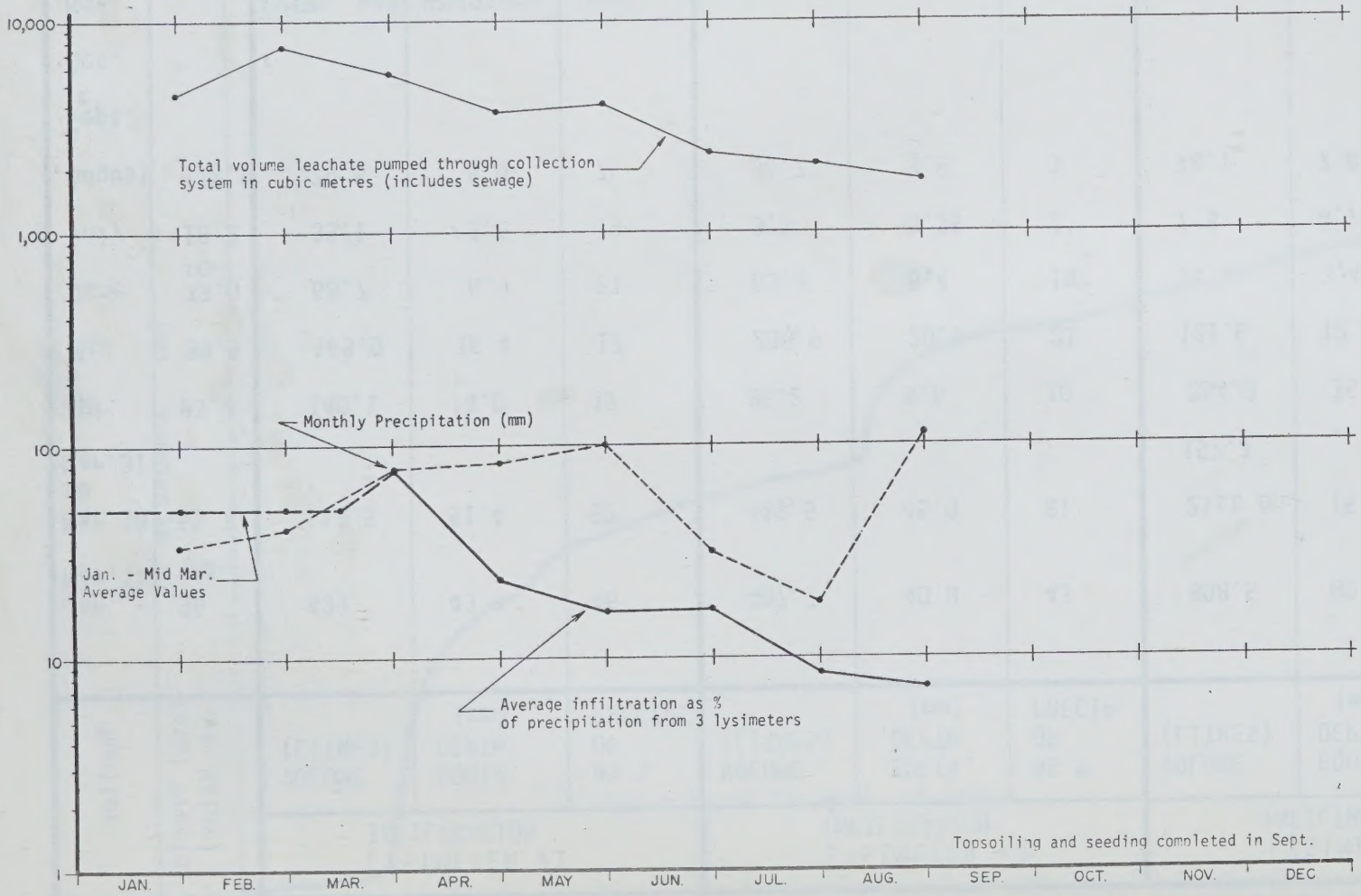
VALUES OF LYSIMETER INFILTRATION, MONTHLY PRECIPITATION AND TOTAL LEACHATE VOLUMES FOR BRITANNIA ROAD LANDFILL SITE



the spring melt period and decreases through the summer. This agrees well with the plot of total leachate which has been pumped through the collection system. However, the values for total leachate volume are not fully representative of the leachate generated by infiltration within the two completed cells, particularly for the period January to March. During this period, Cell 4 was partially excavated with a portion of the underdrain leachate collector system in place and operational. Consequently, all the precipitation falling within the cell would have entered the collection system. Thus, a significant portion of the so-called leachate would have actually been surface water during this time. Landfilling actually commenced in cell 3 in early March and it has been assumed that much of the precipitation falling within the cell was absorbed by the garbage from April onwards.

The total precipitation between January and August was 497.9 mm of water. The average total amount of infiltration from three lysimeters was 136 mm for the same period. This is equivalent to 27% of the total precipitation . An estimate of the total amount of infiltration which occurred through the covers of Cells 1 and 2, based on adjusted leachate volumes, is between 60-120 mm or 14 to 24% of total precipitation. The variance is due to the amount of surface water which is assumed to enter the leachate collection via Cell 4. Even with the uncertainty associated with the leachate volumes, the correlation between the two methods of measuring infiltration is reasonably good. These initial data suggest that the yearly infiltration at a clay-covered landfill site in southern Ontario may well

be in the order of 180 to 200 mm (7 to 8 inches). The equivalent leachate generation rate is 4 Litres per minute per hectare of landfill (0.3 gallons per minute per acre).

Evaluations for 11 full scale landfill sites in West Germany where leachate is collected, indicated that leachate production is dependent on the degree of compaction achieved for the landfill final cover. The results indicated that for landfill sites with a high compaction cover density which was in excess of 0.7 t/m^3, the leachate production rate was within the range of 15-25% of the annual precipitation rate. For those landfill sites classed as having low compaction cover density ie. below 0.7 t/m^3, the leachate production rate was within the range of 25-50% of the annual precipitation rate. The in-situ density at the Britannia Road site was about 2 t/m^3 which would place it within the high compaction category. Since our results to date indicate an infiltration equivalent to between 14 to 27% of precipitation at this site, these data correlate well with the results from West Germany.

TASKS REMAINING:

Two tasks remain outstanding at this site, during this first phase of the study. A calibration exercise has to be completed at the lysimeter installations. A known

amount of water is to be slowly applied to the ground surface such that it all infiltrates into the lysimeter and the response measured at the well. In this manner, an estimate of the efficiency of the lysimeters can be obtained.

Additional monitoring of the lysimeters is planned through the forthcoming winter and snowmelt period in order to provide a full 12 months monitoring data.

Phase 2 Of The Study:

The lysimeter design arising from Phase 1 appears to function satisfactorily such that a similar design can be adopted during Phase 2 of the project. The second phase is intended to measure infiltration through various types of landfill cover materials, located in various physical settings such as geographic location, slope vegetation etc. From here, it should then be possible to compare the actual amount of infiltration with that predicted from the empirical values and drainage equations presently used in landfill cover design.

CONCLUSIONS:

A lysimeter installation to measure the actual amount of infiltration through a landfill cover has been developed and field-tested. Preliminary results indicate that infiltration through a well-compacted clay cover material may amount to about 25% of the total annual precipitation. Although further verification of these data are necessary, the implication is that the yearly infiltration through a clay cover at a landfill site in southern Ontario is likely to be in the order of 180 to 200 mm (7 to 8 inches). The equivalent leachate generation rate is about 4 Litres per minute per hectare (0.3 gallons per minute per acre). With slight modifications this type of lysimeter can be used to assess infiltration through landfill covers with various scenarios of slope, type of material, degree of compaction, vegetative cover and climate types.

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THE USE OF MARSHLANDS IN WASTEWATER TREATMENT

- ONTARIO'S RESEARCH PROGRAMS -

BY

S. A. BLACK

OVERVIEW OF A PAPER FOR PRESENTATION

AT THE

TECHNOLOGY TRANSFER CONFERENCE # 4

RESEARCH ADVISORY COMMITTEE

ONTARIO MINISTRY OF THE ENVIRONMENT

NOVEMBER 29-30, 1983

Abstract

The Use of Marshlands In Wastewater Treatment

S. A. Black

Ministry of the Environment

The use of aquaculture concepts for wastewater treatment is receiving increased attention. Systems studied to date, have included both natural and artificial marshlands, ponds, raceways and other structures based upon various combinations of rooted and free-floating vegetation. The use of both artificial and natural marshlands for the treatment of municipal wastewater is being actively evaluated in Ontario.

This paper defines the current status of marshland treatment technologies and evaluates their potential for routine use in municipal wastewater treatment. In so doing, the paper presents an update of the ongoing research studies at the Listowel artificial marsh facility and outlines details of the Bradford natural marsh study, the Cobalt Artificial Marsh Study and the Port Perry marshland treatment demonstration project.

INTRODUCTION

Use of marshes for year-round treatment of sewage has been under study by the MOE since 1980. Although research facilities include a small scale natural marsh at Bradford, efforts have been primarily focused on the use of artificial systems. A small, multi-celled, artificial marsh at Listowel has provided the basis for development of preliminary design and operating criteria. A more recently constructed experimental marsh facility at Cobalt will provide essential information for implementation of this technology in Northern Ontario. A full-sized demonstration facility is under construction at Port Perry which will process the entire community's wastewater flow of 0.4 MGD.

This paper describes Ontario's ongoing research activities, defines the current status of marshland treatment technology and addresses the potential application of this alternative for wastewater treatment.

1 PROGRAM OBJECTIVES

The primary objectives of the Marshland Study Program are:

a) to determine the capacity of natural and artificial marshes to renovate wastewater on a year-round basis in Northern and Southern Ontario and,

b) to define precise guidelines for the design and operation of marshland wastewater treatment systems.

Several secondary objectives relate to the fundamental understanding of marsh treatment systems and to the development of non-routine operational procedures. These include:

c) an assessment of the role of various storage compartments (soil, plants, litter, micoorganisms) in the removal and ultimate fate of wastewater constituents;

d) an investigation of nuisance organisms generated within the marsh environment, their significance and possible mitigative measures;

e) an assessment of the long-term impacts of wastewater additions on wetlands;

f) an evaluation of the impact of vegetation harvesting on effluent quality and litter accumulation and an assessment of potential uses for the harvested biomass.

2 DESCRIPTION OF STUDY SITES

The following research facilities are currently under study as part of the marshland program:

a) Listowel - An artificial marsh comprised of 5 independent systems has been constructed at Listowel. Three system receive pretreated wastes from an existing lagoon and 2 systems receive effluent from a completely mixed pretreatment aeration cell. This facility is unique in the world because of the flexibility in operating conditions including hydraulic loading, depth and retention times. It also includes two different geometric configurations, namely, serpentine or channelled marshes and open rectangular marshes. Construction was completed in July and sewage additions commenced in August of 1980. Funding for the construction of this facility was provided by the Provincial Lottery.

b) Bradford - This 1400 m^2 natural marsh was physically isolated from interactions with adjacent marsh areas. The facility is fully instrumented to allow detailed measurements of water budgets. Background nutrient fluxes were monitored for 2 years and sewage additions (blend of raw sewage and final effluent from the Bradford STP) commenced in June, 1981.

c) Cobalt - Construction of two small artificial marshes in Northern Ontario was funded by the Ministry of Norther Affairs. One plot was located in a mine tailings basin to establish the potential for conversion of the entire basin to a full-scale marsh wastewater treatment facility for this community. The second plot was built in more representative Northern Ontario soil. Final construction and planting with cattails was completed in July, 1982.

d) Other Sites - Although research studies at the above mentioned study sites will define the wastewater renovation capabilities of marshes, the short study span will not provide information on their long-term capacity (i.e. 10-20 years) to accept wastewaters. Consequently, studies of two wetland sites (Regina Mundi College, Southwestern Region and Sherwood Farms in West-Central Region) which have received pretreated wastewaters over extended periods of time were initiated in 1982.

3 TREATMENT CAPABILITIES

Several research studies throughout North America have demonstrated the viability of marshland treatment of wastewater but to date no design and operating crtieria for routine application of this technology have been developed. Wastewater renovation capabilities documented in scientific literature are highly variable and reflect the different hydraulic loading rates, wastewater characteristics and climatic conditions. Based on a recent worldwide literature review, Reed and Bastian (1980) provide the following broad ranges of removal efficiencies for major wastewater constituents:

Constituent	Range of Removal Efficiency (%)
Suspended Solids (SS)	60 - 90
Biological Oxygen Demand (BOD_5)	50 - 96
Total Nitrogen (TN)	30 - 98
Total Phosphorus (P)	10 - 90

Clearly, further effort is required to define the wastewater renovation capabilities of wetlands and develop reliable engineering criteria.

Although U.S. research efforts precede Ontario's program their studies have focused on the summer application of dilute wastewaters, to natural wetlands. Ontario studies emphasize year-round application of more concentrated wastewaters, to minimize pretreatment costs and eliminate the need for winter storage. Since little effort has been directed, elsewhere, to the use of artificially created marshes, studies at Listowel have generated world-wide interest. A brief synopsis of the results obtained to date at each of the Ontario study sites is provided below:

a) Listowel - The first year of operation at the artificial marsh site in Listowel produced highly variable results. Effluent quality during the first three months superseded the capabilities of even the most advanced conventional processes. A dramatic decline in treatment efficiency during the subsequent winter months coincided with a three fold increase in hydraulic loading rates. Variable results were achieved during the following spring and summer season, despite reduced hydraulic loads.

Clearly, the data generated during the first 12 months period reflect the instability of the newly created marshes, the large initial absorption capacity and the variable hydraulic loadings. Nevertheless, the first year of operation provided valuable insight into the numerous factors which impact on marsh performance and led to effective adjustments in the mode of operation. Throughout this period, it was also apparent that, despite identical operating conditions, the channelled marshes consistently outperformed the open marsh systems. Short-circuiting of wastewaters was identified as the primary cause and design criteria were evolved to address this problem.

The consistent effluent quality achieved during the second year of operation was indicative of a more stable ecosystem. Figures 1 to 5 illustrate the wastewater renovation capabilities of a channelled marsh system (System IV) for this period. The results are presented both in terms of actual concentrations (mg/l) of major constituents in the influent and marsh effluent and in terms of mass balances (g/m^2). The latter values are more representative of marsh performance since the concentrating and diluting effects of evapotranspiration and precipitation are taken into account. This is particularly relevant during prolonged periods of hot, dry weather when evapotranspiration significantly reduces the volume of outflow from the marshes, and thus concentrates the wastewater constituents.

Consequently, high reductions in both BOD and SS were achieved on a year-round basis (Figures 1 and 2). Minor declines in removal efficiencies during ice-free periods reflect the generation and decomposition of marsh biota, particularly phytoplankton. Concentrations of both constituents in the marsh effluent were well below the current 15 mg/l design guidelines for conventional activated sludge secondary plants.

High removal rates for total phosphorus were also sustained throughout the year (Figure 3) and concentrations of this element remained below the 1.0 mg/l objective for conventional processes with phosphorus removal. Maximum phosphorus concentrations in the marsh effluents coincided with the summer period and reflect both the increase in net loading and the concentrating effects of evapotranspiration. With continuous P removal in the pretreatment step, P concentrations of <0.3 mg/l should be achieved. Nitrogen removal rates were far more variable with high removal efficiencies evident only during the summer season (Figures 4 and 5). Elevated ammonia concentrations during the winter months could limit the application of this technology in areas with very restrictive receiving waters. However, a more suitable pretreatment option could eliminate this difficulty.

b) Bradford - Hydraulic loading rates and concentrations of major constituents in the wastewater influent closely parallel the Listowel operation. Mass balance of data, spanning a 7 month period subsequent to sewage additions, show reductions in BOD, SS and TN comparable to the artificial marshes at Listowel. A 60% reduction in P loading is, however, significantly lower than the >80% removal achieved at Listowel. Since the marsh site at Bradford was already enriched with phosphorus prior to sewage addition, its reduced ability to assimilate large quantities of this element is not unexpected.

c) Cobalt - Continuing problems with severe exfiltration from the mine tailings plot (#2) have precluded the establishment of a routine water quality monitoring program. Efforts are still underway to correct the problem. While some information has been generated from the other plot (#1), the data span less than 6 months and are thus extremely preliminary.

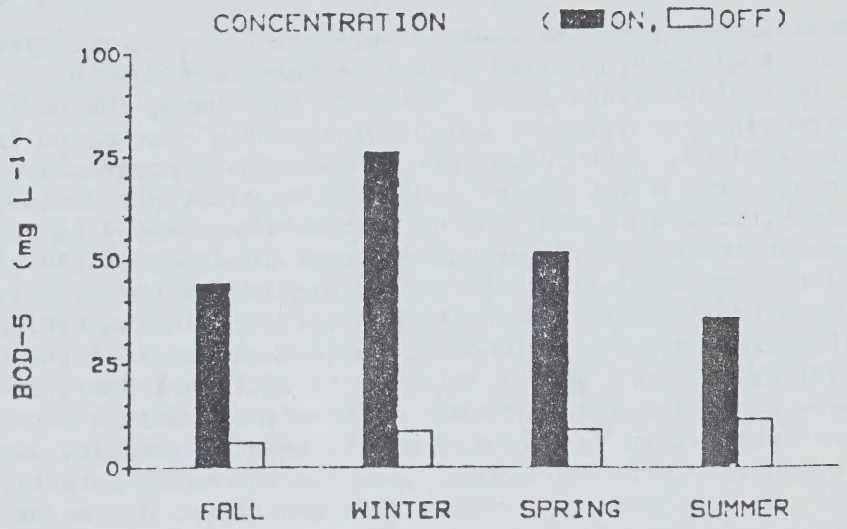
To date, reductions in SS, BOD, TN and P compare favourably with Listowel results, with effluent quality meeting secondary treatment objectives. The raw sewage influent is quite dilute as a result of the deteriorating condition of the community's sewer and water mains. Consequently, higher hydraulic loading rates are being applied at this site.

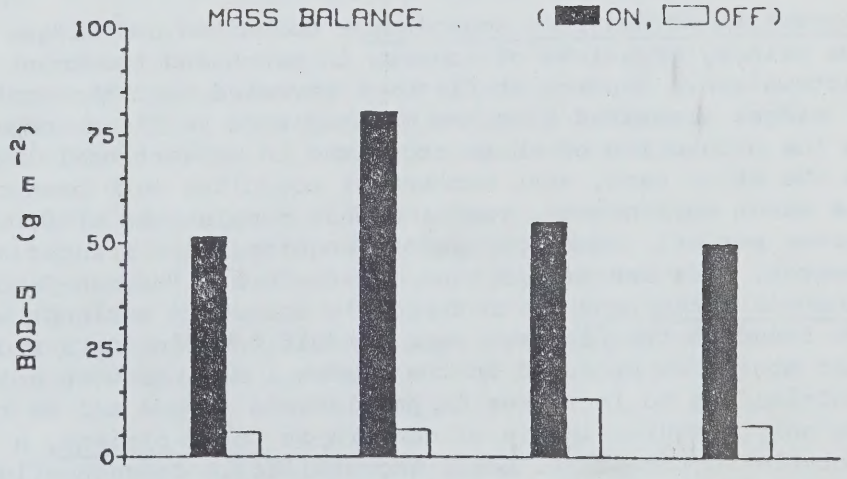
d) Other Sites - A preliminary survey of Ontario wetlands which have received wastewater effluents over an extended period of time (>10 years), revealed only two sites which were considered suitable for further study.

Wastewaters from an extended aeration plant at the Sherwood Farm Poultry Processing Operation have been discharged to a 1 ha wetland since 1972. Prior to this date, the wastes were piped to a lagoon which subsequently discharged into the wetland. Historical air photos show a progressive change from a wet meadow ecosystem to a cattail dominated marsh, following addition of wastewaters. Based on company production records, current effluent loadings of 178 m^3/day to the marsh appear to be representative of the past 15 years. Concentrations of wastewater constituents in the effluent discharged to the wetland are quite variable (5.8 to 10.3 mg/l P; 0.3 to 5.5 mg/l NH_3: 3.9 to 28.7 mg/l BOD). Historical data on effluent quality are extremely sporadic. Wastewaters are retained in the marsh for only brief intervals, suggesting extensive short-circuiting. Despite the brief retention time, and the many years of wastewaters additions, all major wastewater constituents are reduced by some 40% during passage through the marsh.

A second study site, adjacent to Regina Mundi College in London, receives effluent from a contact stabilization plant, built in 1963. The entire marsh/pond covers an area of 8 ha. The small wastewater loading of 50 m^3/day was found to be insignificant when compared to other sources of input such as runoff from the watershed area. Consequently, information gathered from this site is of little use in assessing long-term impacts of wastewaters on wetlands.

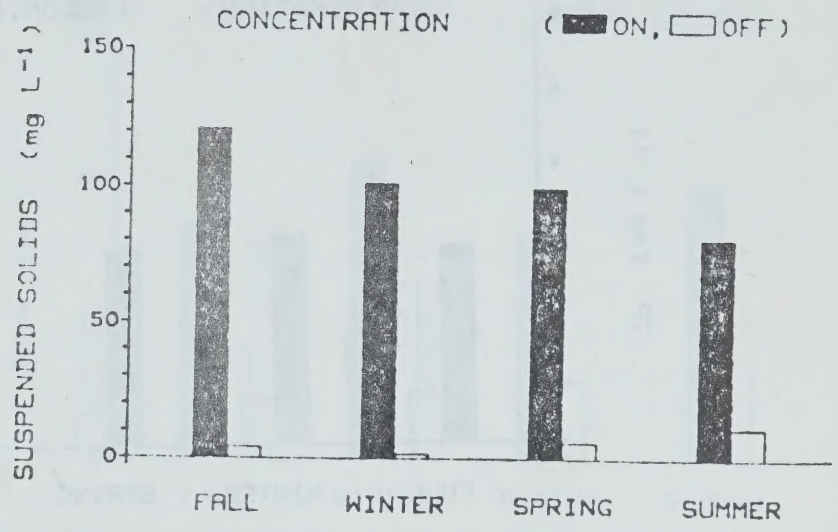
e) Generation of Nuisance Organisms - Mosquitos and midges are the primary organisms of concern in marshland treatment of wastewaters. Studies at Listowel revealed that the numbers of midges generated from the marshes were small in comparison to the production of these organisms in conventional lagoons. On the other hand, vast numbers of mosquitos were produced in the marsh environment, reaching peak populations of 9,000 larvae per m^2. Four species of mosquitos were identified, however, none are significant man-feeders. The man-feeding Mansonia perturbans which typically occurs in wetlands was not found at the Listowel site. Adult trapping data showed that mosquitos produced in the Listowel marshes were not contributing to increases in populations beyond a 1 km radius. The only mosquito specie of concern is Culex pipiens, a probable vector of St. Louis Encephalitis. Consequently, chemical control would only be required in the event of Encephalitis activity. A recently registered bacterial insectide, Bacillus thuringiensis which provided 90% control in trials at Listowel, is recommended for this purpose.





	FALL	WINTER	SPRING	SUMMER
% REMOVAL	88.4	91.7	75.4	84.9

FIGURE 1: REDUCTION IN BOD-5 IN SYSTEM 4, 1981-1982.



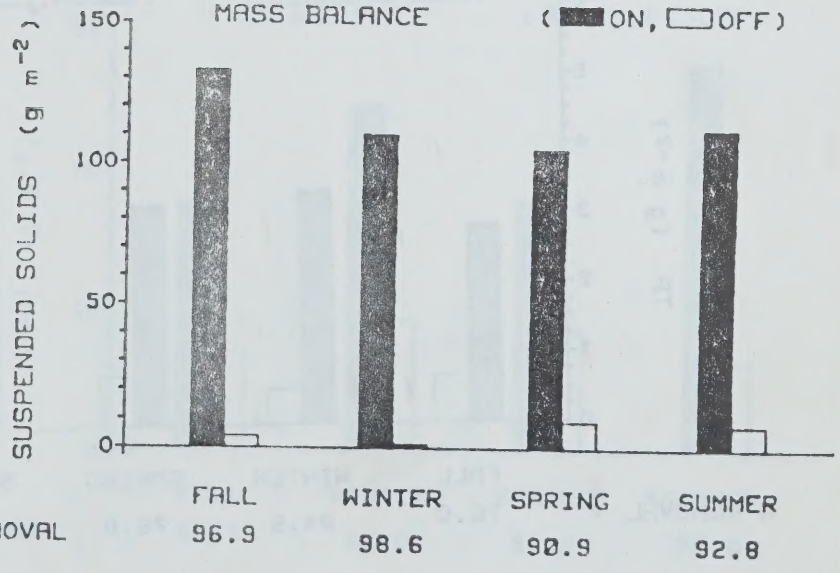
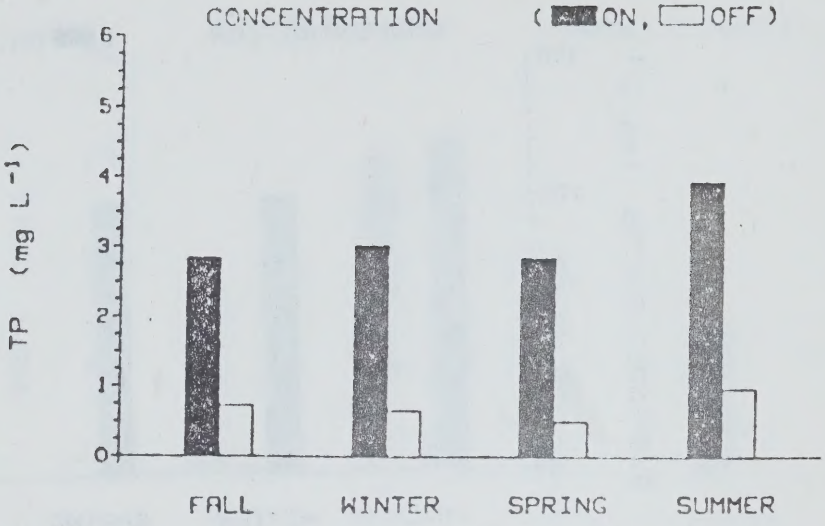
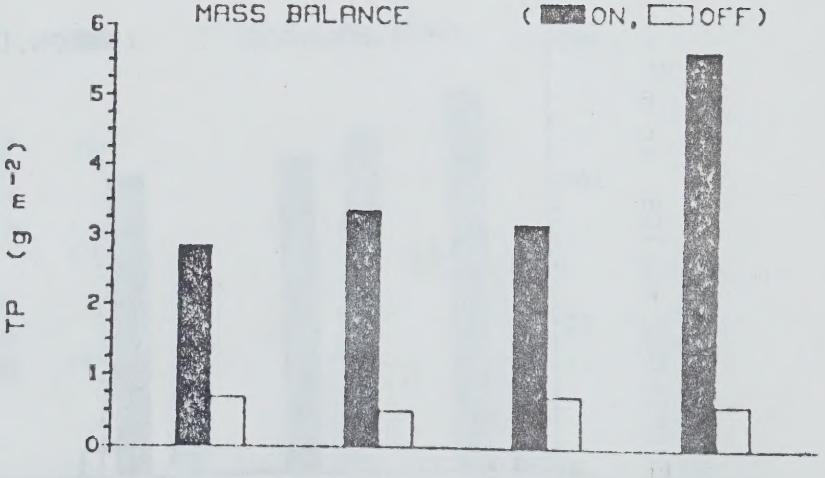


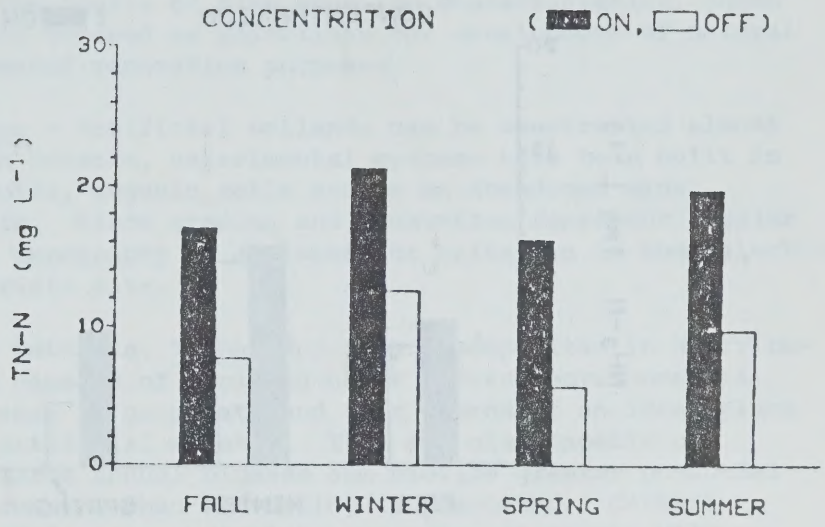
FIGURE 2: REDUCTION IN SUSPENDED SOLIDS IN SYSTEM 4, 1981-1982.





	FALL	WINTER	SPRING	SUMMER
% REMOVAL	76.0	84.6	76.8	89.1

FIGURE 3: REDUCTION IN TOTAL PHOSPHORUS IN SYSTEM 4, 1981-1982.



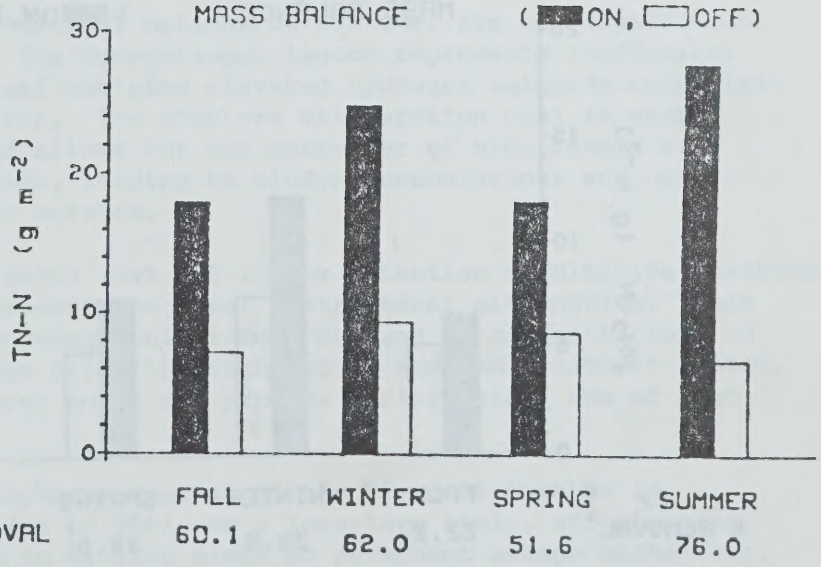
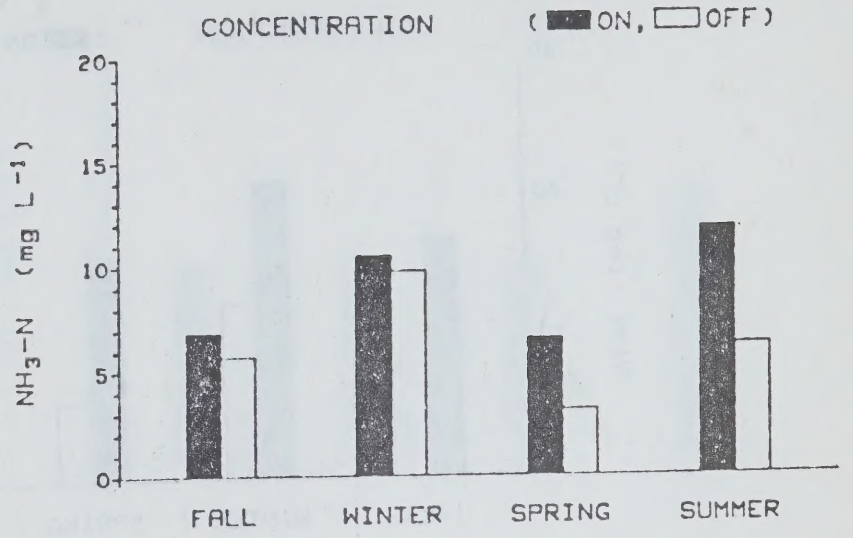
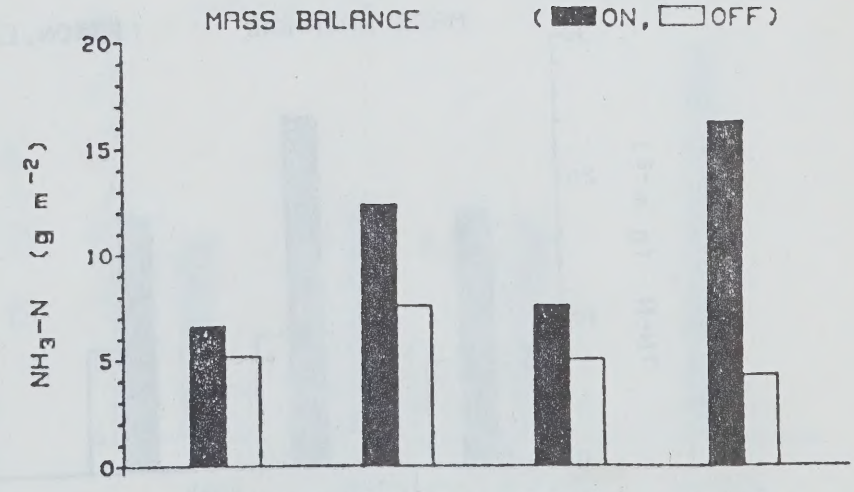


FIGURE 4: REDUCTION IN TOTAL NITROGEN IN SYSTEM 4, 1981-1982.





	FALL	WINTER	SPRING	SUMMER
% REMOVAL	22.2	38.5	33.9	73.9

FIGURE 5: REDUCTION IN AMMONIA IN SYSTEM 4, 1981-1982.

4 PRELIMINARY DESIGN CRITERIA

Based on studies at Listowel, the following preliminary design criteria have been developed for artificial marshland treatment systems. Within the limits of site specific characteristics, these criteria could also be used as guidelines for development of natural marshes for wastewater renovation purposes.

a) Site Selection - Artificial wetlands can be constructed almost anywhere. In Ontario, experimental systems have been built in heavy clay soils, organic soils and in an abandoned mine tailings basin. Since grading and excavating represent a major cost factor, topography is an important criterion in the selection of an appropriate site.

b) Vegetation - Cattails, Typha. spp. are cosmopolitan in distribution, hardly capable of thriving under diverse environmental conditions, easy to propogate and thus represent an ideal plant species for artificial marshes. They are also capable of producing a large annual biomass and provide greater potential for N and P removal when harvesting is practised. Cattail rhizomes planted at approximately one meter intervals will produce a dense stand within three months in Southern and Central Ontario. Planting at reduced densities or merely allowing for natural invasion of vegetation will achieve the same objective but over a more extended period of time.

c) PreTreatment of Raw Sewage - To reduce costs, minimal pretreatment of wastewater prior to discharge to a marsh is desirable. The level of pretreatment will, however, influence the quality of the final marsh effluent and therefore effluent objectives must be taken into consideration.

The two pretreatment options at Listowel are now regarded as inadequate. The conventional lagoon represents inefficient use of land and contains elevated hydrogen sulphide concentrations in winter. The complete mix aeration cell is energy intensive and allows for the carryover of high levels of volatile solids, leading to sludge accumulations and oxygen stress in the marshes.

It is anticipated that a 5-10 day retention facultative aeration cell may represent the ideal pretreatment alternative. This option should adequately reduce BOD and SS concentrations in the raw sewage prior to discharge to a marsh treatment system, minimize energy costs and provide for efficient use of land resources.

Although phosphorus removal in the Listowel marshes is substantial (up to 98%), on a long-term basis, efficiencies are expected to decline since no permanent escape mechanisms, such as denitrification exists for this element. Therefore, phosphorus reduction by chemical addition is recommended in the pretreatment step.

d) Marsh Configuration - Studies at Listowel have demonstrated the significance of the geometric configuration of an artificial marsh to performance efficiency. In large open marshes, short-circuiting of wastewater will occur, leading to reduced treatment efficiency. Consequently, marsh systems should be designed with length to width ratios equal to or greater than 10:1 or alternatively with internal baffling to ensure proper flow distribution.

e) Hydraulic Loading - Results obtained at Listowel suggest that hydraulic loading rates of 200 m^3/ha/day of pretreated wastewater will produce a high quality effluent. Further testing is required to assess marsh performance at higher hydraulic loadings.

f) Hydraulic Detention Time - For optimal performance, a 5 to 10 day detention period for wastewater within the marsh is required. At a constant hydraulic loading, detention time will be influenced by two major factors, namely, evapotranspiration in summer and ice formation in winter. High evapotranspiration rates will increase detention times and cause stagnation and severe anoxia, whereas ice formation will decrease detention time. Maintaining low summertime liquid depths (<10 cm) and raising water levels in the marsh (>30 cm) prior to the onset of winter conditions will remedy these problems. Consequently, artificial marshes must be designed to provide flexibility in the regulation of liquid depths.

g) Land Requirements - At a loading rate of 200 m^3/ha/day, a 1 MGD (4,546 m3/day loading of wastewater would require some 60 acres (24 ha) of marsh and additional land for internal flow regulation and pretreatment facilities. This compares favourably with conventional seasonal discharge lagoons which require some 100 acres (40 ha) for an equivalent sewage loading.

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THE HAMILTON STUDY: RELATIONSHIP BETWEEN OUTDOOR AND INDOOR AIR QUALITY IN HOMES AND ELEMENTARY SCHOOLS

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Measurements have been made of indoor and outdoor levels of nitrogen dioxide (NO_2), sulphur dioxide (SO_2) and suspended particulate material (SPM) using a multipollutant sampler which we have developed (1). Observations were made for two 5-day sessions at each site, once during the heating season (H) and once during the non-heating season (NH). Paired indoor and outdoor observations were made during the same 24-hour sampling period at each site using the same instrumentation. Complete sets of paired data were obtained for 16 schools in both seasons, for 20 homes in the non-heating season and 14 of that 20 in the heating season. Data were much more difficult to obtain from homes than schools. The study showed patterns of results which were systematic for each pollutant, but differed between pollutants. Most of the results reported here will be in terms of indoor-outdoor ratio (I/O) expressed as percent, in the non-heating or heating season. For NO_2, in schools the I/O ratio was close to 75%, and was found to be independent of heating season, with indoor levels strongly correlated with outdoor levels. In homes, the I/O ratio depends on the presence of indoor sources, such as smokers, or gas stoves. In the absence of these, there is relatively little effect of heating season, and the ratio is about 80%. Smokers were not found to have an effect on indoor NO_2 levels in either season, but gas stoves were associated with markedly increased ($P < 0.001$) levels of NO_2 in both seasons, contributing an additional 13 ppb (NH) and 25 ppb (H). This resulted in homes with gas stoves having an I/O ratio of 1.8 (NH), 2.1 (H).

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* The final report is presently under review. For more information, please contact the author.

For SO_2, in schools, the I/O ratio was about 22%, independent of heating season, indoor levels being correlated with outdoor levels ($P<0.01$). In homes, the indoor level depends on heating season, and is poorly correlated ($P>0.05$) with outdoor levels in either season. The I/O ratio = 64% (NH), 15% (H) (SE 13%). For SPM, in schools, I/O ratio was 90% (NH), 140% (H), but there was no significant correlation with outdoor levels or season. In homes, I/O ratio was 134% (NH), 101% (H). In the non-heating season there was no significant correlation with outdoor levels, or the presence of smokers. In the heating season, there were significant effects from outdoor levels and smokers. The presence of smokers added 35 $\mu g/m^3$ of particulate to indoor levels.

The results of this study show that in schools, both for NO_2 and SO_2, indoor levels are well correlated with outdoor levels, independent of heating season, whereas indoor particulate levels are variable, frequently higher than outdoor levels, and uncorrelated with outdoor levels or heating season.

In homes without indoor sources for NO_2, there is little effect of heating season, and the indoor levels are correlated with outdoor levels. Gas stoves are a major indoor source, giving rise to indoor concentrations double the outdoor concentrations, during both heating and non-heating seasons. Levels of SO_2 are low indoors, poorly correlated with outdoor levels, and strongly dependent on heating season, being much lower during the heating season. Suspended particulate material levels indoors were generally higher than outdoors, and poorly correlated with outdoor levels in either season. During the heating season, however, the presence of smokers contributed substantially ($P<0.01$) to the particulate load, but had no effect on the NO_2 or SO_2 levels.

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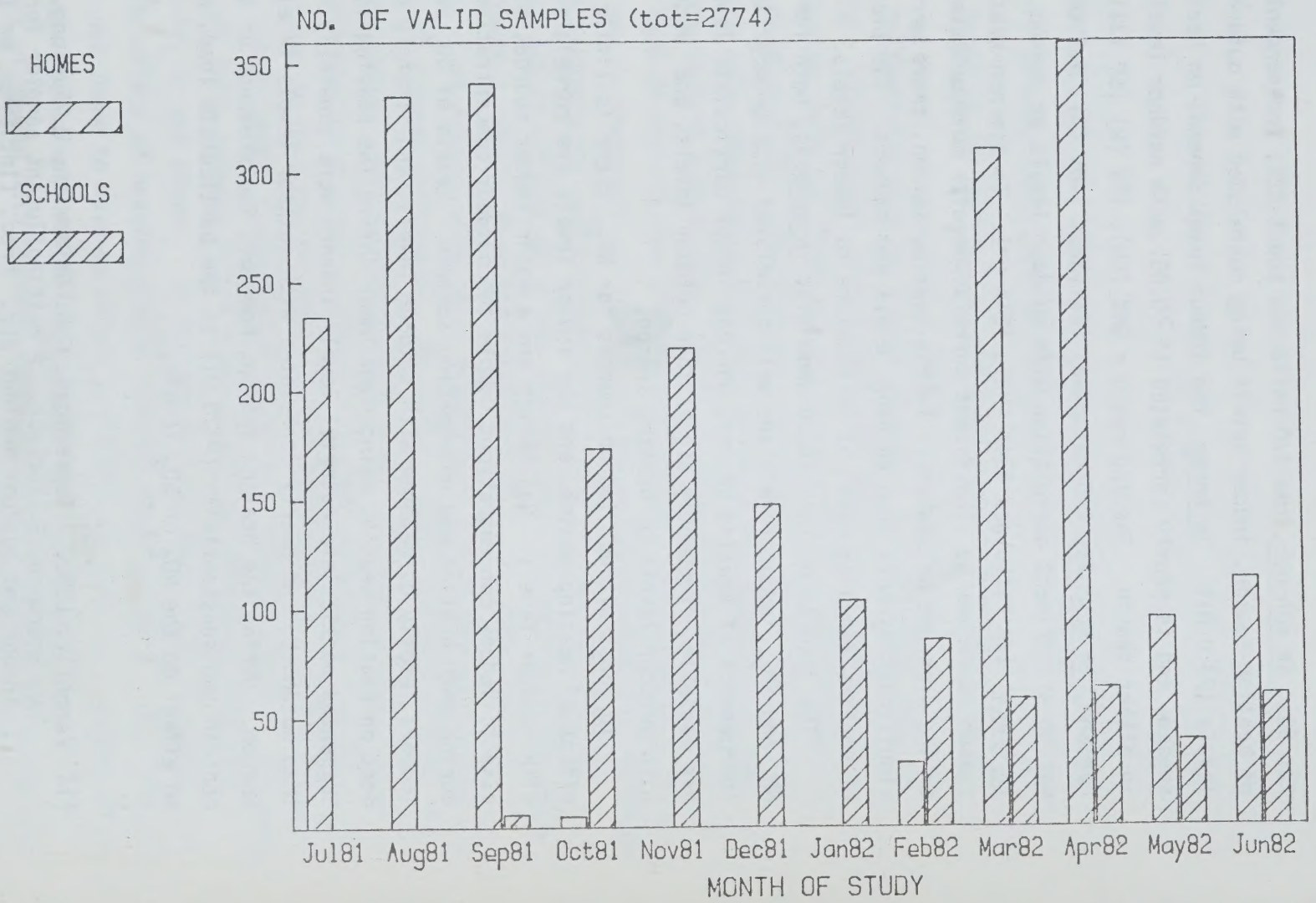
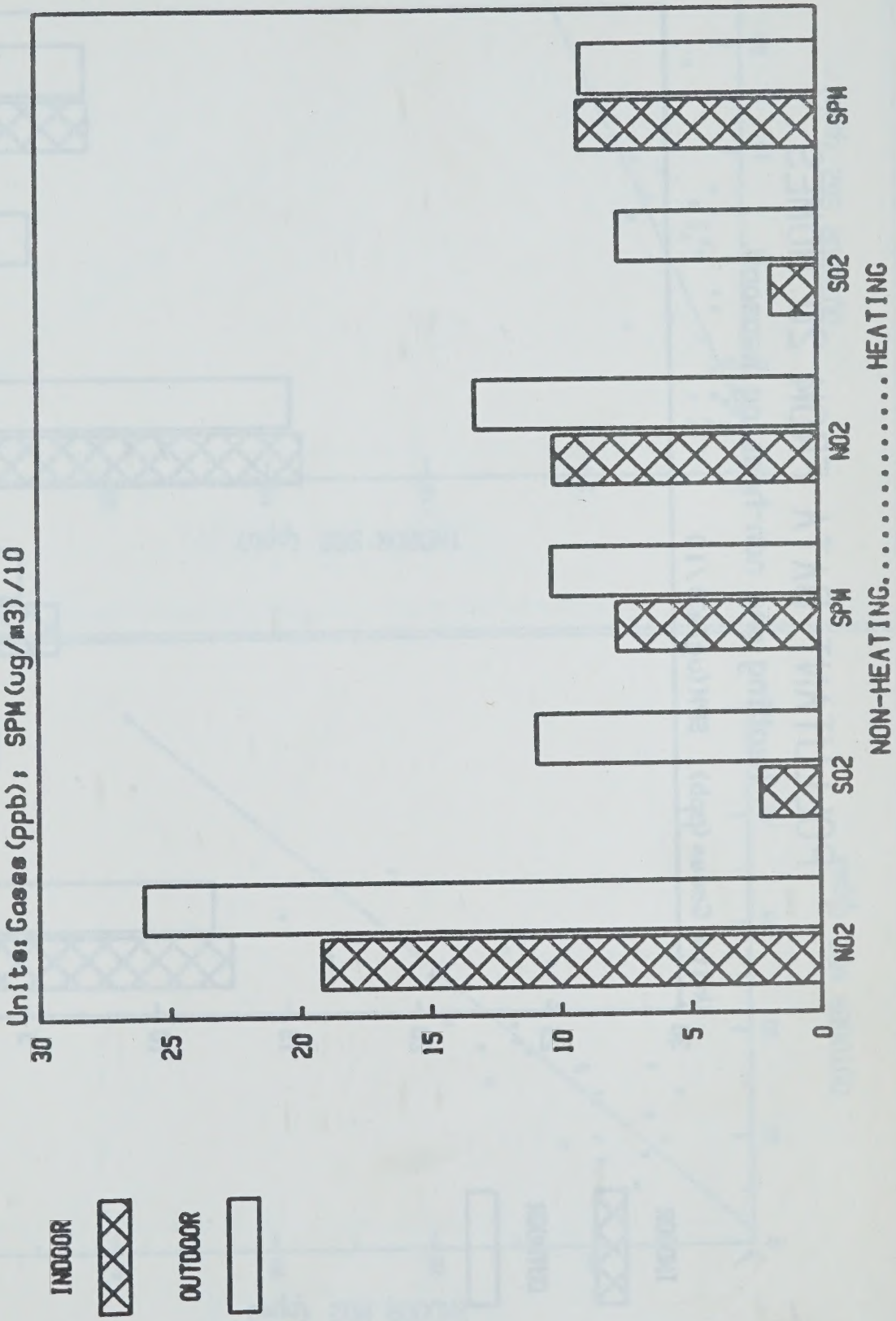
I/O STUDY: VALID SAMPLES

For both homes and schools.




POLLUTANT DATA FROM 16 SCHOOLS
heating and non-heating seasons.
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30
25
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NO2
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NO2
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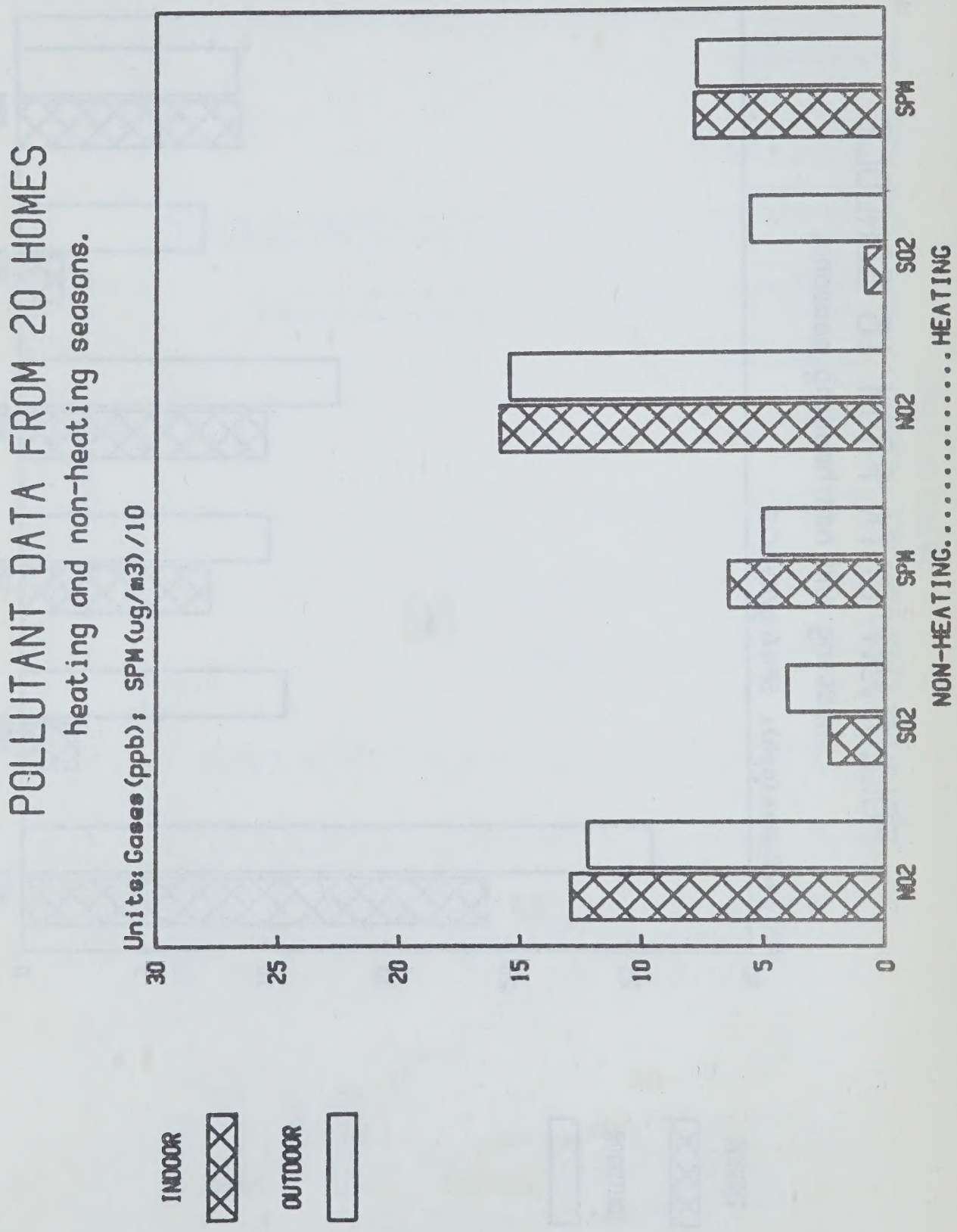
NON-HEATING.....HEATING
INDOOR
OUTDOOR


POLLUTANT DATA FROM 20 HOMES
heating and non-heating seasons.
Units: Gases (ppb); SPM (ug/m3) /10
INDOOR
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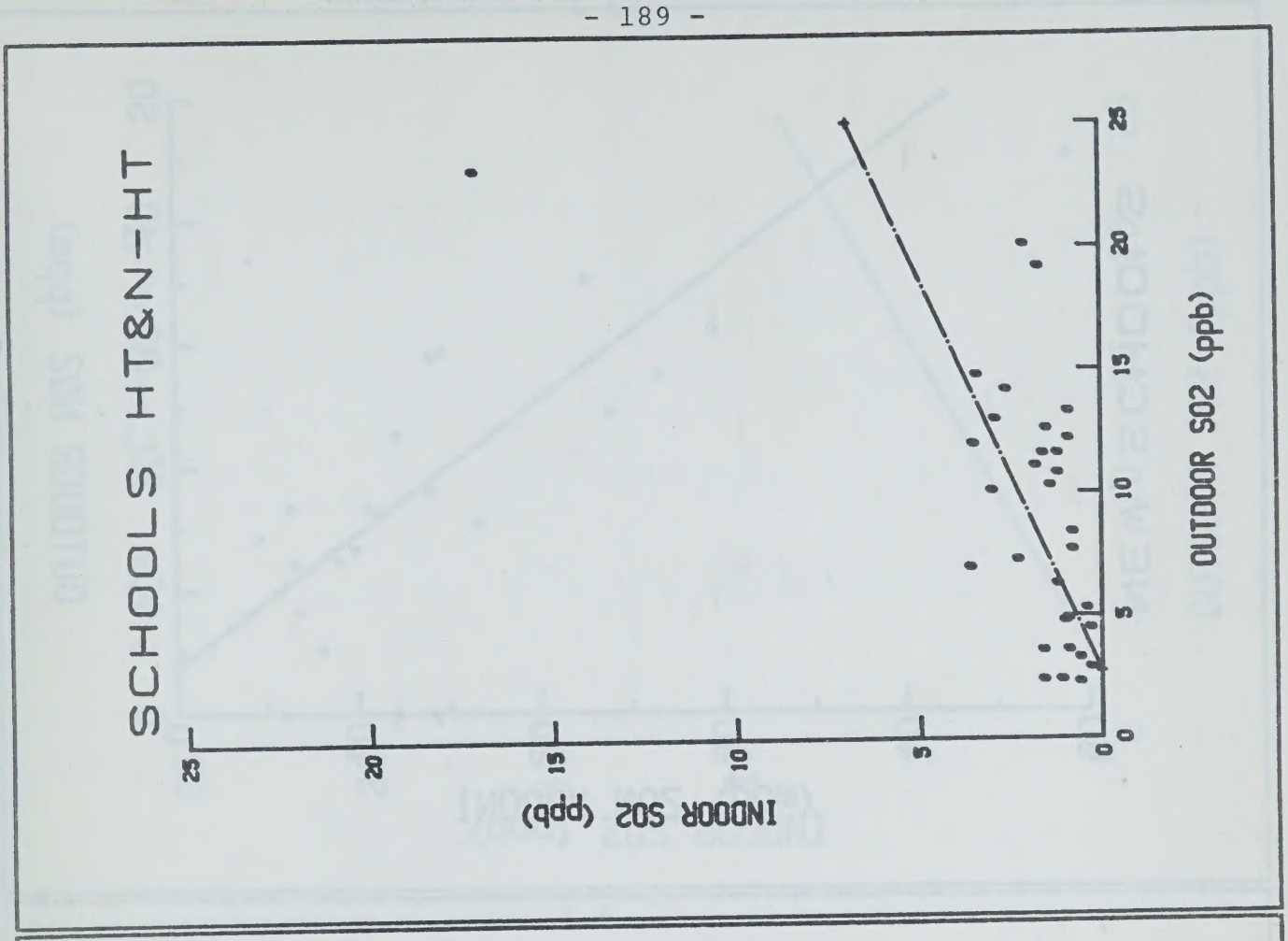
SCHOOLS HT&N-HT
INDOOR SO2 (ppb)
OUTDOOR SO2 (ppb)
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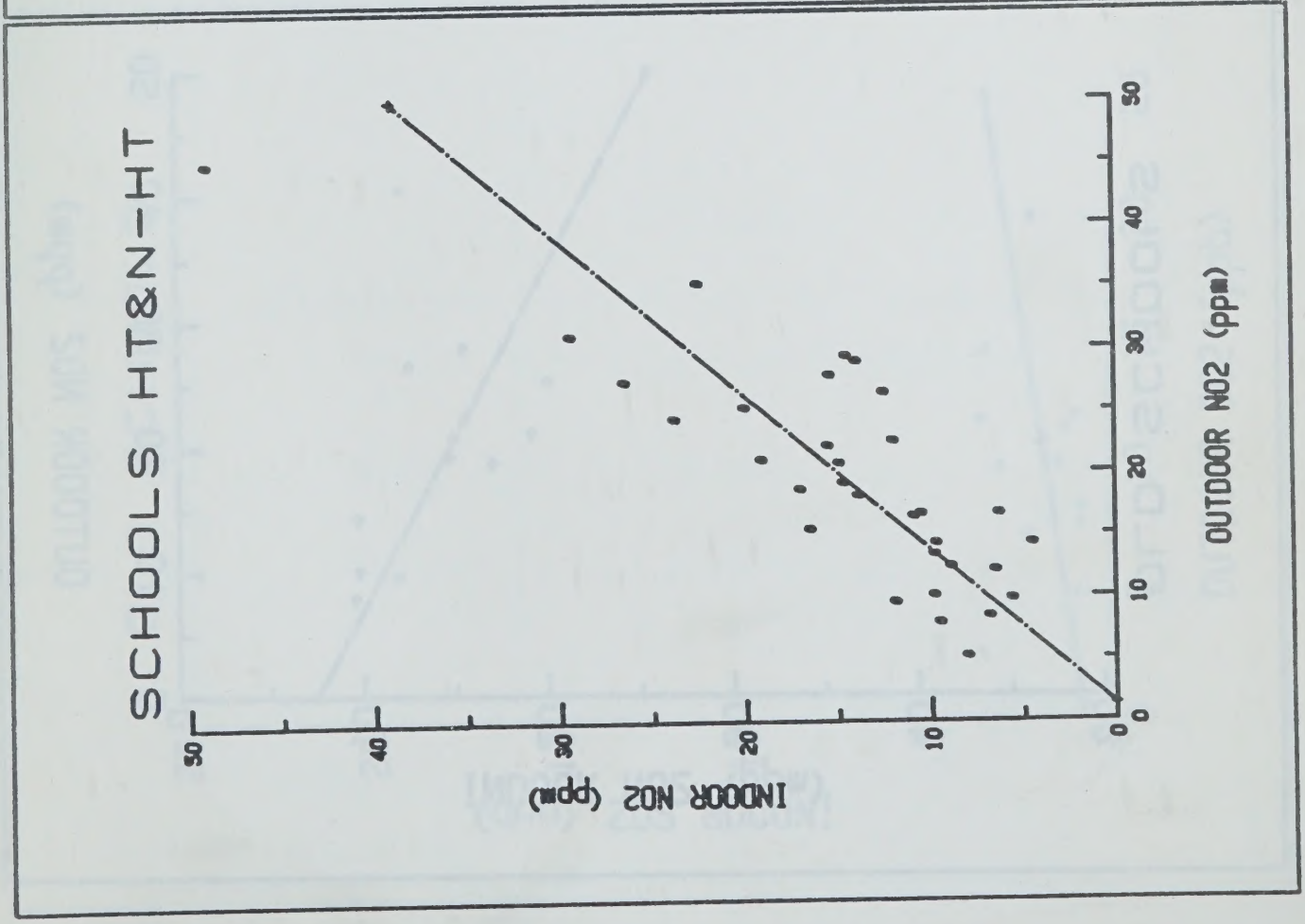
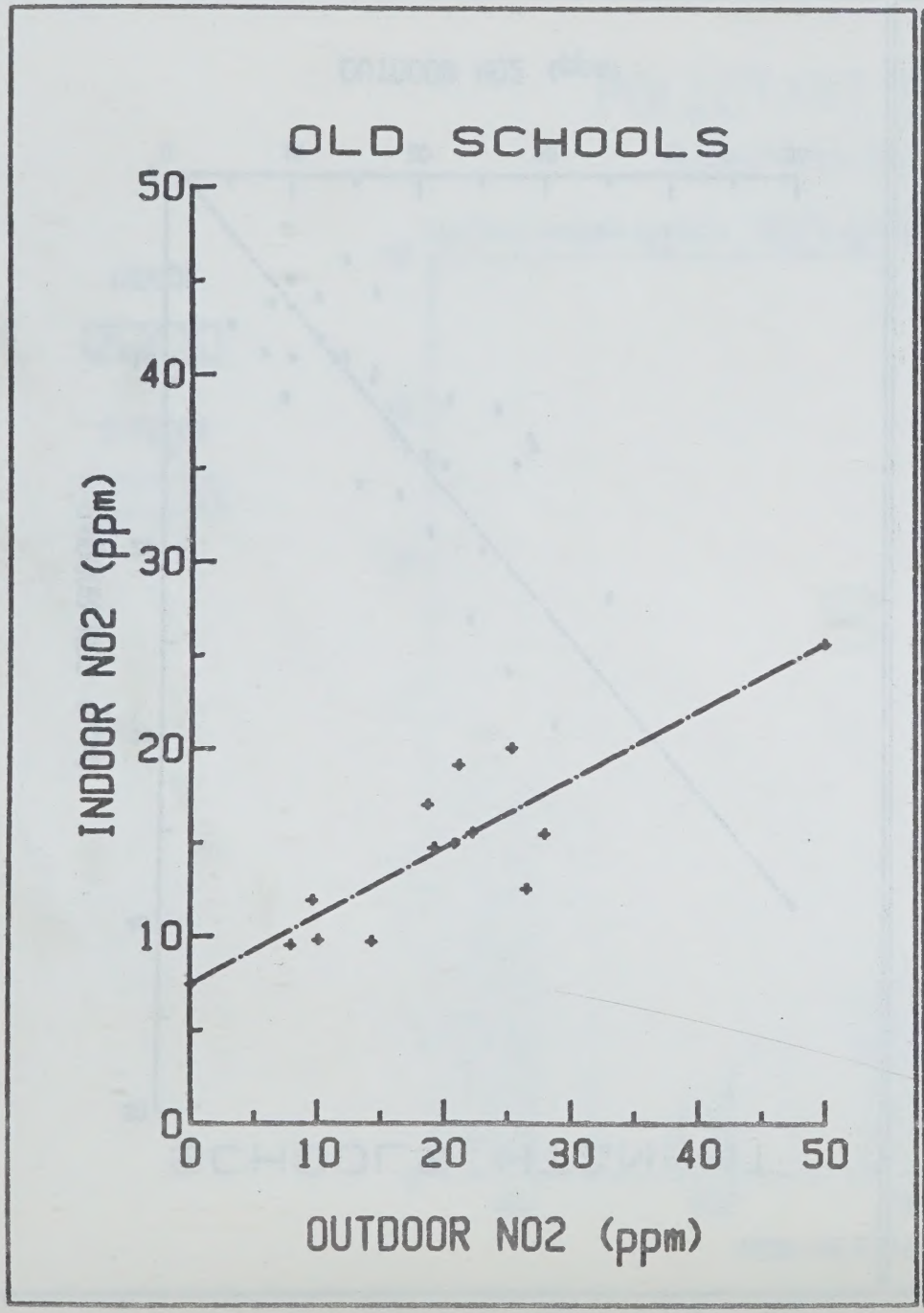
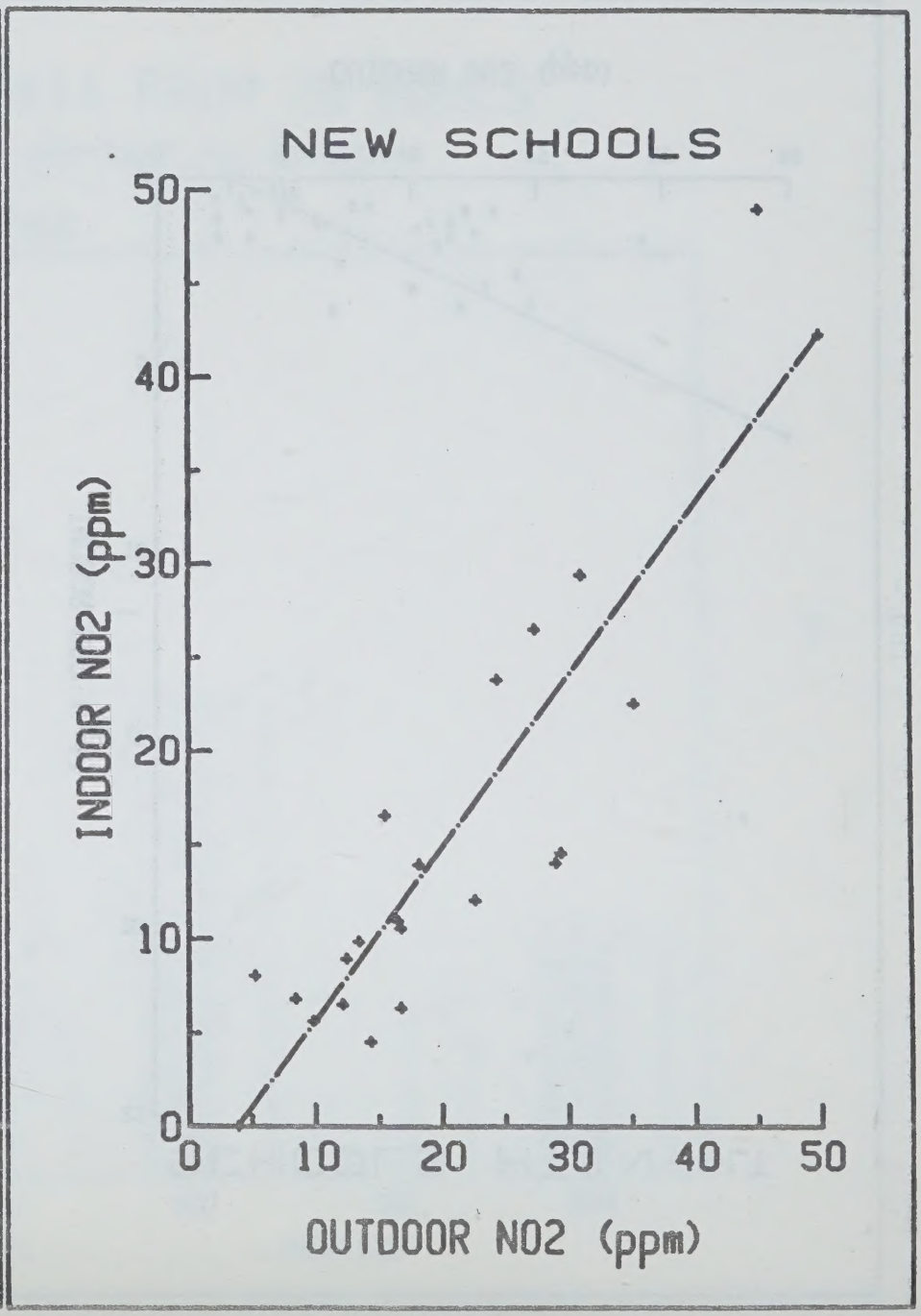
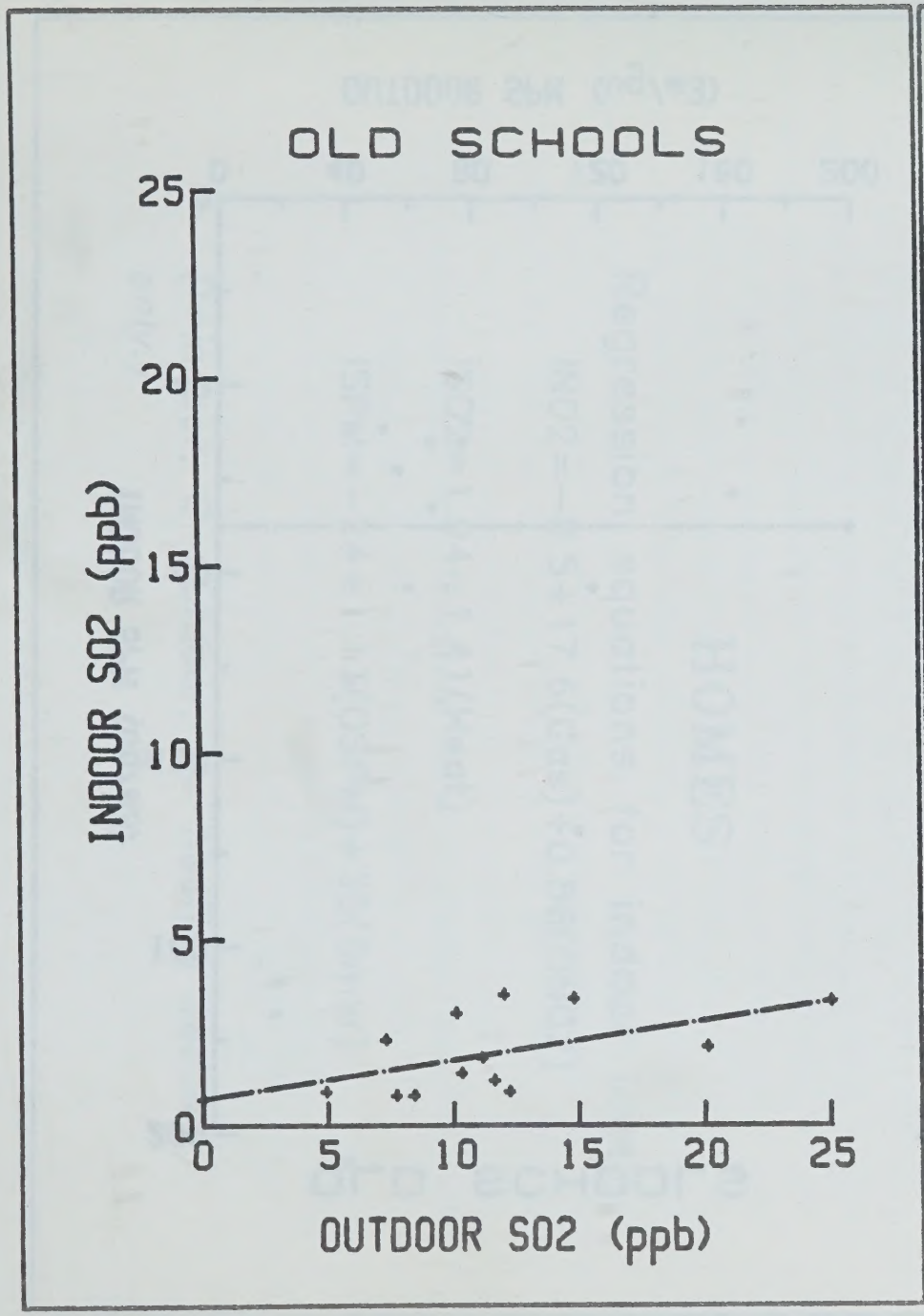
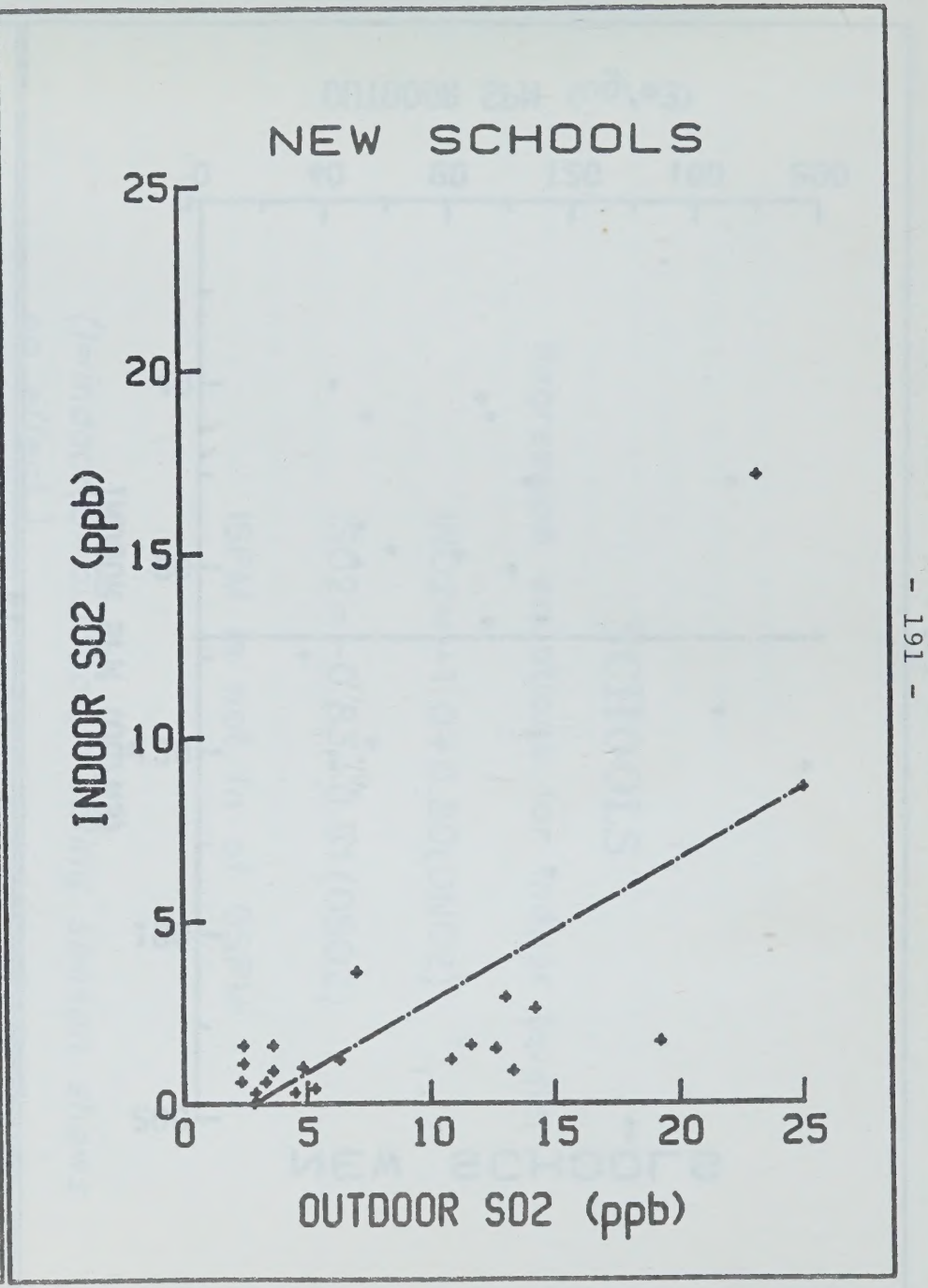
5
10
15
20
25

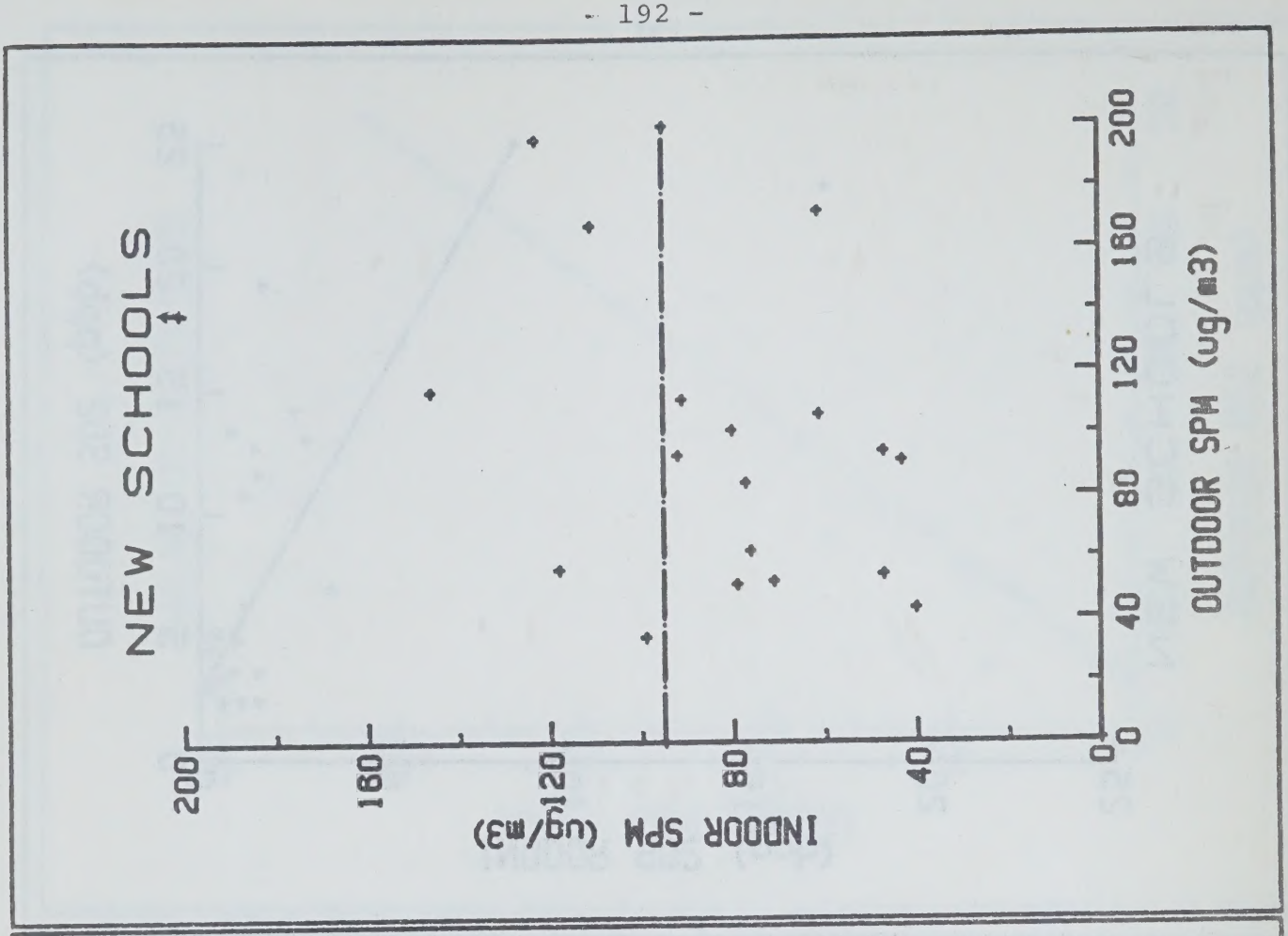

SCHOOLS HT&N-HT
INDOOR NO2 (ppm)
OUTDOOR NO2 (ppm)
0
10
20
30
40
50

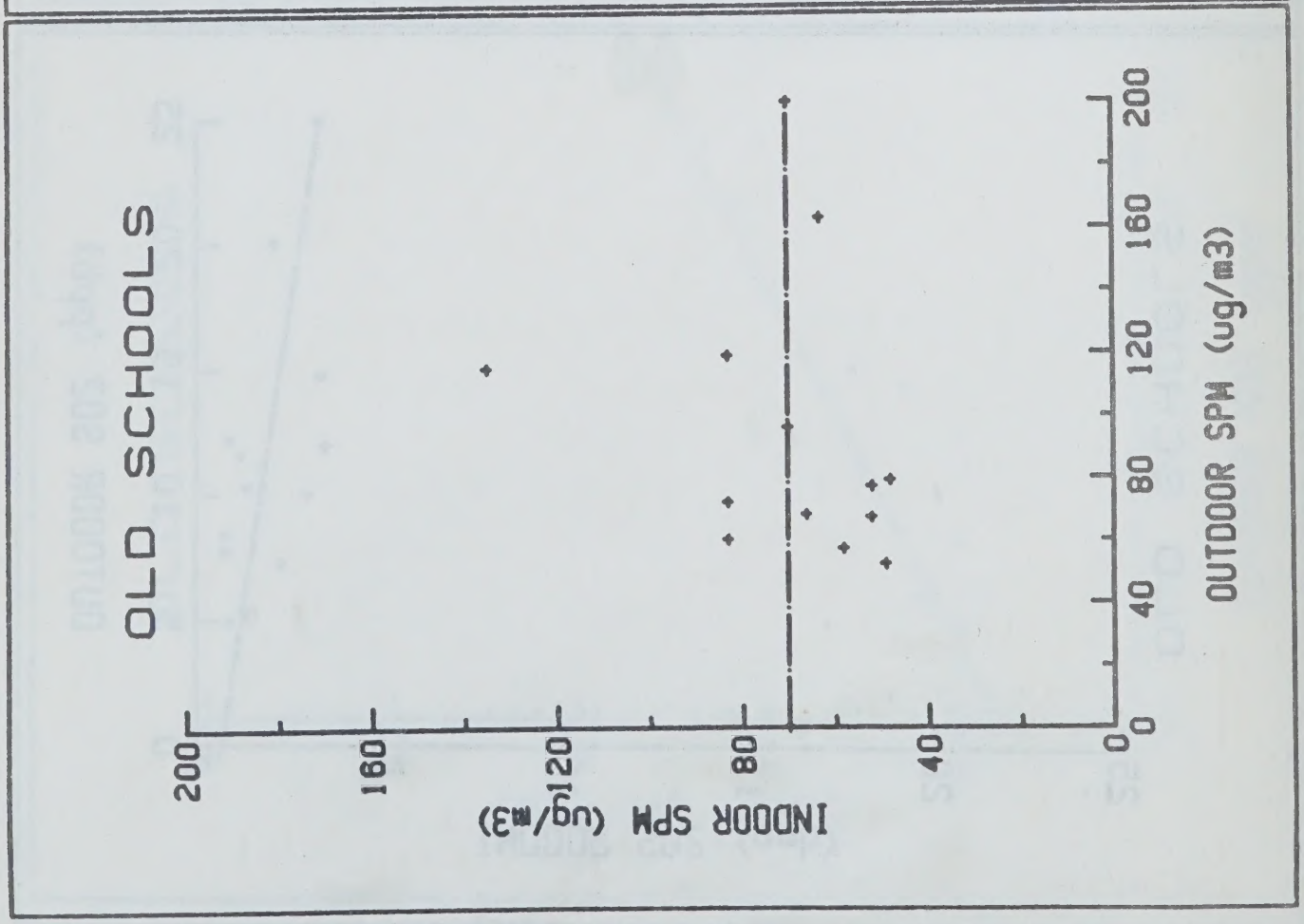

OLD SCHOOLS
INDOOR NO2 (ppm)
OUTDOOR NO2 (ppm)
0
10
20
30
40
50


NEW SCHOOLS
INDOOR NO2 (ppm)
OUTDOOR NO2 (ppm)
0
10
20
30
40
50


OLD SCHOOLS
INDOOR SO2 (ppb)
OUTDOOR SO2 (ppb)
0
5
10
15
20
25


NEW SCHOOLS
INDOOR SO2 (ppb)
OUTDOOR SO2 (ppb)
0
5
10
15
20
25


NEW SCHOOLS
INDOOR SPM (ug/m3)
OUTDOOR SPM (ug/m3)
0
40
80
120
160
200


OLD SCHOOLS
INDOOR SPM (ug/m3)
OUTDOOR SPM (ug/m3)
0
40
80
120
160
200

SCHOOLS

Regression equations for indoor levels:

$$INO2 = -1.0 + 0.80(ONO2)$$

$$ISO2 = -0.83 + 0.31(OSO2)$$

ISPM is not fn of OSPM

(I=indoor,O=outdoor; heating season shows no effect.)

HOMES

Regression equations for indoor levels:

$$INO2 = -0.5 + 17.6(Gas) + 0.86(ONO2)$$

$$ISO2 = 1.94 - 1.41(Heat)$$

$$ISPM = -24 + 1.13(OSPM) + 35(Smkr) \quad *$$

*(I=indoor, O=Outdoor; *= heating season only.)*

COMBINED APPLICATION OF OZONE AND CHLORINE OR CHLORAMINE TO REDUCE PRODUCTION OF CHLORINATED ORGANICS IN DISINFECTION OF HIGH DOC DRINKING WATERS

J.L. Robertson[1]

ABSTRACT

The application of ozone as the primary disinfectant was investigated on water supplies in Ontario containing high concentrations of dissolved organic carbon (DOC), as a means to reduce elevated levels of trihalomethanes and other chlorinated organics. Laboratory and pilot plant studies demonstrated that the use of ozonation to replace pre-chlorination resulted in substantially reduced THM and NPOX concentrations with subsequent post-chlorination, as compared to conventional pre- and post-chlorination. THM and NPOX production were found to be negligible if ozonation was combined with chloramination as the secondary disinfectant. Batch tests showed that these conclusions applied to another high DOC water supply, although the THM and NPOX production per unit of DOC varied between water supplies. This demonstrated the need for pilot studies on individual water supplies to develop data for process design. Preliminary cost estimates for various sizes of ozonation plants were prepared to assess the general economics of adding ozonation to existing water treatment plants. The study concluded that ozonation, combined with controlled post-chlorination or chloramine application, represented an effective strategy for control of THM and NPOX in high DOC waters while maintaining bacteriological quality and providing colour reduction.

Keywords: ozonation, chlorination, chloramination, trihalomethanes, chlorinated organics, disinfection, drinking water, cost estimates

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INTRODUCTION

The role of chlorination in drinking water disinfection is being re-examined as a result of the discovery of trihalomethanes in Dutch drinking water by Rook (1974). Subsequently, trihalomethanes were measured in North American drinking water by Bellar and Lichtenberg (1974), by Symons et al. of the USEPA (1975), by Nicholson et al. of the Ontario Ministry of the Environment (1976) and Health and Welfare Canada (1977). Ontario MOE data (1977) indicated that ten of forty-two municipalities had total trihalomethane levels in their drinking water in excess of 100 ug/L in the summer months, as measured by the direct aqueous injection (DA1) method. Chloroform has been declared a potential carcinogen and bromo-chloro-methanes have been found to be mutagenic. This resulted in the promulgation of a 100 ug/L limit on total trihalomethanes in drinking waters by the U.S. EPA in the 29 November 1979 Federal Register.

Conventional water treatment utilizes chlorination for a variety of purposes including:

- primary disinfection of raw water
- taste and odour control (occasionally through super-chlorination)
- control of bacterial and algal growths within clarifiers and filters
- colour removal
- destruction of ammonia (breakpoint chlorination)
- provision of distribution system residual

Where the raw water supply contains high concentrations of organic carbon (eg. humic materials) or high algal concentrations, chlorination of the raw and treated waters will result in the formation of trihalomethanes and other organo-halogen compounds. As many of these compounds represent a potential health hazard, their minimization or elimination is considered to be beneficial to public health.

There are a variety of water treatment options available for preventing the formation of part or all of the THM and organo-halogen compounds. These include the following options singly or in combination:

- elimination of pre-chlorination or change in point of application
- THM precursor removal by coagulation and/or direct filtration
- use of alternate disinfectants such as ozone, chlorine dioxide, or chloramine
- precursor removal by GAC or ozone/GAC.

Where raw water quality is such that pre-disinfection cannot be eliminated, use of alternate disinfectants is a feasible method for minimizing or eliminating THM and TOX formation. This will occur with raw waters having high DOC and poor bacteriologic/algal quality.

IEC BEAK has completed a study for the Ontario Ministry of the Environment to investigate the technical and economic feasibility, and some of the public health aspects, of ozonation to reduce the concentrations of trihalomethanes in drinking water by replacing chlorine in the primary disinfection process. The project involved the use of both bench and pilot scale water treatment systems to evaluate ozone application for the primary or pre-disinfection step, and considered both chlorine and chloramine for the secondary or post-chlorination step.

A detailed description of the experimental program was presented in 1982, at the Technology Transfer Conference No. 3. In this paper, some of the important technical aspects of the project are reviewed, together with cost estimates for "add-on" ozonation systems, and some practical considerations for the application of ozonation.

PROJECT DESCRIPTION

As indicated, a detailed description of the project has been already presented. The overall approach to the project is described below to provide background information for the presentation of the key technical results of the project.

Objectives

The specific objectives of this program were:

- to demonstrate the effectiveness of ozonation as a water tretment step for controlling the production of trihalomethanes and other chlorinated organics in water treatment.

- to investigate the practical methods which might be employed in water treatment plants to reduce the formation of undesirable by-products.

- to identify as many of the organic by-products of ozonation of surface water supplies as possible.

- to assess the public health effects of ozonation with respect to disinfection and mutagenic activity.

- to assess the add-on cost of providing ozonation at existing water treatment plants.

Approach

The project was undertaken in two phases to examine the feasibility of using ozone in Ontario for the treatment of drinking water supplies.

Phase 1 consisted of the following major tasks:

- literature review
- bench scale batch experiments on application of ozone followed by chlorine or chloramine
- mutagenicity assessment of the organic by-products of ozonation, ozonation/chlorination, and ozonation/chloramination
- identification of organic by-products by GC/MS and HPLC/UV fluorescence.

Raw waters from three Ontario municipalities were used in the Phase 1 experimental work to evaluate the potential for both the formation and the minimization of trihalomethanes and other chlorinated organics in the disinfection process. Brantford and Lindsay were selected as they represented water supplies from an industrialized area, and rural area respectively, with high DOC. Toronto was also selected because it draws water from Lake Ontario which serves as a water supply for a large portion of Ontario's population and had a low DOC.

In Phase 2, the following major tasks were completed:

- literature review
- seasonal pilot-scale testing of ozone followed by chlorine or chloramine to confirm the results of Phase 1 under field conditions
- preparation of cost estimates for providing add-on ozonation at existing water treatment plants.

The demonstration pilot plant was set up at Lindsay, Ontario. Lindsay municipal water is drawn from the Scugog River, which flows from a relatively marshy area. The DOC is high, typically 8 to 12 mg/L, due to the presence of natural organic material in the raw water. Typically, water temperatures may vary from freezing to 25°C during the year. Colour in the raw water typically varies from about five units in the winter to as high as 50-60 units in the summer. High bacterial and algal counts occur during the summer months with some taste and odour.

The factors which were evaluated in both the bench scale and pilot scale programs had been determined from the literature and were similar for both programs. These factors, together with the pilot plant conditions, are presented below:

Factor	Levels (Pilot)
o DOC	o normal variation - 6 to 12 mg/L
o ozone dosage	o 4 levels - 0, 2.0, 7.5, 20 mg/L
o delay time between ozonation and subsequent chlorine residual application	o 3 levels - 3, 6, 24 hours
o storage time after chlorine residual application	o 3 levels - 4, 24, 72 hours
o type of residual applied	o 2 levels - hypochlorite or chloramine
o seasonal effects of water quality including temperature	o 3 seasons - winter, summer, fall

For the pilot scale test program, these factors were set up in incomplete factorial experimental designs for each season to facilitate statistical analysis. Details of the approach were published in the MOE Technology Transfer Conference No. 3 proceedings.

TECHNICAL ASPECTS OF OZONATION

Disinfection with Ozone and Hypochlorite

Statistical analysis of the experimental data from the pilot plant ozonation studies yielded the following results at the 95% confidence level.

- o delay time between ozonation and subsequent chlorine residual application had no significant effect on THM production
- o storage time after chlorine residual application had no significant effect on THM production
- o the application of ozone as the primary disinfectant significantly reduced THM production although dosage had no significant effect in the range of 2-20 mg/L of ozone applied.

As a consequence of the first two results, it was possible to pool the THM data from all test runs at each applied ozone dosage to obtain the mean chloroform production data shown in Table 1. The data from Table 1 are presented graphically in Figure 1.

Table 1 and Figure 1 illustrate that the use of pre-ozonation reduces chloroform production but that ozone dosage had little effect on the magnitude of the reduction. Up to 60% reduction in chloroform could be achieved by replacing conventional pre-chlorination/post-chlorination with pre-ozonation/post-chlorination, when a 30 minute chlorine residual was applied. Figure 2 presents the chloroform data for the measured raw water temperatures to illustrate the reductions in chloroform production possible from the elimination of pre-chlorination and its replacement by pre-ozonation.

The lack of effect of delay time and storage time on chloroform apparently resulted from the high DOC and associated high chlorine demand. While each sample contained a residual of 0.5 mg/L of free chlorine after 30 minutes, this residual was partially exhausted and the remainder converted to chloramine residual, within the shortest storage time of 4 hours. A total chlorine residual of 0.1-0.2 mg/L remained in all samples up to the longest storage time of 72 hours. In effect, the amount of free chlorine was insufficient to meet the demand from 4, 24, or 72 hours storage thus limiting the amount of chloroform produced.

This has significant implications for the practical application of ozonation to provide properly disinfected water from high DOC raw water sources having high coliform counts and potential taste and odour problems. In previous research, THM formation potential was determined by the addition of an excess of free chlorine and observing the THM production with time. It was in this manner that previous researchers determined that the effectiveness of ozonation for reducing the production of THM decreased with increasing delay time between pre-ozonation and post-chlorination.

In practice, however, the delay time is dependent on the amount of treatment required to purify the raw water (eg. coagulation and filtration) and cannot be varied appreciably. The results of this study demonstrate that limiting the free chlorine available for reaction, prevents the increased THM production resulting from increased delay time and storage time observed in other studies. Provided that the high bacteriological quality of the treated water can be maintained, ozonation followed by controlled or limited chlorine application represents an effective strategy for reduction of THM. This could be achieved by the application of a free chlorine residual with a short contact time prior to addition of ammonia to form chloramine. This would provide a lasting residual and would not react readily to form THM or other chlorinated organics.

The NPOX data presented in Table 2 show that low applied ozone dosages (2 mg/L) reduced NPOX production by 50% to 60%, but as higher ozone dosages were applied NPOX production increased. This would be expected if it was assumed that the destruction of existing organic sites for chlorination resulted from low ozone dosages, and was followed by generation of new sites as a result of the action of higher dosages on the organic matrix. This assumption implies a sharp decrease in chlorine demand at low applied ozone dosages followed by a gradual increase in chlorine demand with increasing applied ozone dosages. The chlorine demand data showed such a pattern. When NPOX was plotted against chlorine demand, a significant linear relationship was found.

Disinfection with Ozone and Chloramine

A parallel set of experiments using chloramine as the secondary disinfectant following ozonation showed that no chloroform or other THM were produced during post-chloramination. A fresh chloramine solution prepared with distilled water was used to produce a 0.5 mg/L total chlorine residual in the ozonated water samples. In practice,

low concentrations (< 10 ug/L) of THM may be formed during the addition of chlorine to a water containing natural organics used as feedwater to the chlorinator.

Measurement of NPOX production in the same experiments with chloramine showed that the use of chloramine instead of chlorine reduced the NPOX levels to < 10 ug/L.

Loss of residual during storage was not a problem with chloramine as it had been with hypochlorite. Starting at a total residual of 0.5 mg/L after 30 minutes, total residuals remaining at 4 and 24 hours were in the range 0.15 to 0.35 mg/L. This is adequate for bacteriostatic purposes (Rich, 1963). Residuals of 0.05 to 0.15 mg/L after 72 hours storage were measured indicating a need for higher dosages to provide adequate residual protection.

For the purpose of controlling THM and NPOX production in high DOC water supplies, the combination of pre-ozonation and post-chloramination appears to offer the opportunity to minimize or eliminate these classes of compounds. When considered in light of control of the taste and odour and colour removal by ozonation, the advantages appear even greater.

Effect of Water Supply

Batch ozonation tests were performed on Lindsay raw water as well as on raw water from the cities of Brantford and Toronto. The purpose of these tests was to determine the effect of water supply on THM and NPOX production. Chlorine and chloramine dosages and application procedures were similar to those used in the pilot scale testing. Ozone was applied according to an ozone/DOC ratio to ensure that equivalent ozone conditions were used for each water supply. The ratios used were 0.2, 0.75 and 2.0, which corresponded to the 2, 7.5 and 20 mg/L dosages applied at Lindsay in the pilot testing. The pooled THM and NPOX data are presented in Tables 3 and 4 respectively.

The THM Production from DOC (ug/mg) data were calculated by summing the ug/L concentrations of the four trihalomethanes $CHCl_3$, $CHCl_2Br$, $CHBr_2Cl$ and $CHBr_3$ and dividing by the DOC in mg/L. Several observations may be made as follows:

- different water supplies appeared to produce different quantities of THM per unit of DOC when subjected to chlorination alone and ozonation/chlorination,
- ozonation/chlorination produced less THM than did chlorination alone,
- chloramine produced negligible THM and very low concentrations of NPOX in all water supplies, either alone or in combination with ozone
- ozonation/chlorination appeared to produce less NPOX than did chlorination alone.

The differences in production of THM and NPOX in different high DOC water supplies demonstrate the importance of conducting pilot tests prior to design of an ozonation facility, to determine the extent of THM and NPOX reduction achievable.

Public Health and Aesthetics

Total and fecal coliform counts were performed on samples of raw and pre-ozonated water collected during the pilot plant testing. Table 5 presents the mean total coliform data for raw and pre-ozonated water grouped by season and ozone dosage. No data on viruses were collected.

It is evident from Table 5 that pre-ozonation at all dosages resulted in effective destruction of total coliform. It appears, therefore, that the turbidity present in the raw water (1-10 JTU) did not adversely affect the disinfection of the raw water by ozone. Complete destruction of fecal coliforms was also observed.

True colour measurements were made with a comparator in all three seasonal surveys. On the basis of the comparator results presented in Table 6, it is apparent that significant reduction of true colour was obtained through pre-ozonation as has been observed in previous work.

The data for March indicated greater than 75% removal of colour, to a level below the MOE drinking water criterion of 5 units at all ozone dosages. However, in July colour could be reduced only by 50% to 65% to between 10 and 15 units depending on ozone dosage. Even at 20 mg/L of ozone the maximum colour removal averaged 65% and left residual true colour of 10 units in the ozonated water. This is consistent with observations by previous researchers who observed that in highly coloured natural waters, a refractory portion of the colour remained after ozonation.

The results of the organic identification work by GC/MS and HPLC showed that ozonation produced a wide variety of new compounds. Most of these may be rationalized in terms of oxidative cleavage of the natural humic materials in the raw waters. Higher concentrations of these materials resulted from higher ozone dosages, as expected, and also from higher DOC concentration in the raw water. Lindsay water, which had the highest DOC, also yielded the largest number of ozonation by-products. Many of the by-products were ketones, aldehydes or organic acids presumably formed from the cleavage of carbon-carbon double bonds by the classical Criegee mechanism. Several aromatic carboxylic acids were found, as well as many substituted phenols. These presumably were formed by cleavage of the natural aquatic humic materials.

In general, the compounds identified were in the low parts-per-billion concentration range or less, and their combined concentrations represent a small fraction of the largely uncharacterized matrix of organic carbon compounds found in natural waters. Most of the organic ozonation products found are similar to products obtained by natural oxidation processes and are probably innoccous.

The results of Ames' salmonella testing for bacterial mutagenicity showed essentially no mutagenic activity from the organics isolated by a vacuum rotary evaporation procedure. The rotary evaporation procedure recovers the non-volatile organic materials from water which have boiling points greater than 100°C. This represents in excess of 90% of the organic matter present in water supplies. The results in this study have been interpreted to indicate that this large fraction of the organics in raw and treated waters are not mutagenic under the test conditions applied in this study.

ECONOMICS OF OZONATION

To provide realistic cost estimates for ozonation systems in Ontario, a variety of plant sizes and ozone dosages had to be considered. Application of pre-ozonation most likely will be considered for water supplies having high DOC concentrations and hence high THM's, taste and odour problems, excessive colour and/or low bacteriological quality. On the basis of the pilot testing conducted in this study, and from literature information on taste and odour, ozone dosages of between 2.0 and 7.5 mg/L will provide the desired treated water quality. Consequently these two dosages were used to estimate costs for ozonation systems which could be added on to existing water treatment facilities.

Capital and operating costs for intermediate dosages can be estimated from the range of costs provided.

Water treatment plants in Ontario range from less than 400 m^3/day (100,000 USgpd) to over 200,000 m^3/day (50 MUSgpd). Because of the capital costs of ozonation systems, very small municipalities are unlikely to consider ozonation except in extreme circumstances. Consequently, plant sizes of 1900 m^3/day (500,000 USgpd), 19,000 m^3/day (5.0 MUSgpd) and 190,000 m^3/day (50 MUSgpd) were chosen representing populations of approximately 5,000, 50,000 and 500,000.

Detailed capital and operating costs for these plant sizes were calculated based on "add-on" ozonation systems for hypothetical existing plants. As such, these estimates should not be considered applicable to all water treatment plants of similar capacity. All costs are in 1982 dollars.

Capital costs included direct costs such as building construction, contact tanks, water piping, ozone process equipment, electricals and overhead costs including design and site engineering, contract administration, bonds and permits insurance and contingency. It was assumed that land would be available at existing plants. Budgetary costs were obtained for major items from suppliers and contractors.

Annual operating costs were calculated and included process energy, building energy, labour and maintenance material. Process energy (electrical) was assumed at $0.03/kw-hr.

Total annual costs were calculated from the capital and operating costs as shown in Table 7. These were then converted to a cost per cubic metre of water treated for comparison to existing water rates for various municipalities shown in Table 8.

Cost of "add-on" ozonation ranged from $0.06/$m^3$ for the 7.5 mg/L dose at the 1900 m^3/day plant, to $0.007/$m^3$ for the 2.0 mg/L dose at the 190,000 m^3/day plant. Water rates appeared to be independent of plant size for the municipalities surveyed, so an average water cost of $0.29/$m^3$ ($1.08/1000 USgal) was used. On this basis, addition of ozonation would increase water rates by 20 to 25% for a small plant to 2.5 to 4.5% for a large plant, assuming the cost is passed on direct. For municipalities, such as Lindsay,

where there is a significant annual cost for chlorine, (dosages as high as 10 mg/L) a credit would be realized from the displaced chlorine consumption. This credit would pay for the ammonia needed for chloramination as well as help to offset the annual cost of ozonation.

APPLICATIONS OF OZONATION

Where raw water quality is such that pre-disinfection cannot be eliminated, use of alternate disinfectants is a feasible method for minimizing or eliminating THM and TOX formation. This study examined the use of ozone as an alternate primary disinfectant in combination with both chlorine and chloramine as secondary disinfectants.

As ozonation provides no residual for bacteriostatic action in the distribution system a persistant residual is applied after treatment to last through the distribution system. The results of the pilot studies presented in this report indicate that either short-term chlorination or chloramination could provide the necessary residual and reduce or eliminate THM and chlorinated organics. Selection of short-term chlorination or chloramination for final disinfection will depend on:

- level of THM's achievable by short-term chlorination
- organic nitrogen content of treated water available to provide combined chlorine residual if chlorination is used.

Ozonation has been shown in this study to provide substantial reduction in THM formation by elimination of the pre-chlorination step and by destruction of precursor materials. The data showed that the reductions in THM concentrations achievable with ozonation are largely unaffected by ozone dosages in the range 2-20 mg/L, although there is a trend to lower THM concentrations with increasing ozone dosage.

The decision to use ozonation should be based on consideration of a number of factors and how these are related to the specific water treatment needs and economic considerations at any given water treatment plant. For example, pre-ozonation will reduce or prevent the formation of THM and NPOX when followed by controlled chlorination or chloramination; pre-ozonation will simultaneously destroy or reduce colour depending on the water supply, and provide excellent bacterial destruction. The

City of Monroe, Michigan has had excellent results using pre-ozonation to control taste and odour problems. The actual dosages applied will depend on the purpose of using ozonation and will need to be evaluated in pilot scale tests. In general, however, factors other than THM control will influence dosage selection.

The evidence available from this study and the literature, indicates that ozonation itself generates fewer potentially harmful by-products than chlorination, and appears to destroy most potentially harmful compounds when applied in concentrations used in water treatment. The Salmonella assay results from this study indicate no mutagenic activity of the non-volatile organics in ozonated water, while the reduction of the THM indicates a beneficial health effect. It should be emphasized that the Ames' test data presented in this report represent only the first step in the rigorous procedures needed for risk assessment.

Based on the results presented, complete elimination of THM and NPOX could be achieved through the use of pre-ozonation and post-chloramination. The literature and the Ames' test results in this study indicate that the ozonation/chloramination system would provide the maximum public health benefit in terms of good micro-biological quality and almost complete elimination of bacterial mutagenic agents such as THM and other chlorinated organics.

Because of the capital costs involved in applying both ozone and chlorine dioxide, extensive bench tests and full-scale pilot trials are recommended to determine if acceptable THM and TOX levels can be achieved by modifications of the conventional processes first. If taste, odour and colour removal are also necessary, then alternate disinfectants must be considered at the outset to achieve control of these problems as well as THM and TOX. In general, high DOC raw waters will have colour taste or odour problems associated with them in the warmer months of the year.

Extensive testing programs are recommended to establish the most feasible approach to achieving THM and NPOX removal, while maintaining treated water quality. Tests should be undertaken to establish which of the options for THM and NPOX removal is the most economical, is technically feasible and meets drinking water quality objectives.

When water quality considerations dictate the use of alternate disinfectants, pilot scale trials of ozonation and chlorination/chloramination should be undertaken, similar to those presented in this study.

The pilot trials should be undertaken under "worst case" raw water quality conditions, as these will result in the maximum THM production for comparison to drinking water quality objectives. Worst case conditions would occur mainly in mid-to-late summer when water temperature and DOC are at maximum.

Although no effect was observed for delay time between ozonation and post-disinfection at Lindsay, the delay time used in the tests should be equivalent to that which would occur in the full-scale facility. Similarly, the storage time after chlorination/ chloramination should be based on the retention period in the water distribution system. In this manner realistic THM and TOX data will be obtained on which to base decisions regarding choice of short-term chlorination with or without subsequent ammonia addition, or direct addition of a chloramine residual.

CONCLUSIONS

The following conclusions have been drawn from the study:

1. Ozonation has been shown to provide a substantial reduction in THM and NPOX production when combined with controlled post-chlorination. However, the reduction was largely independent of applied ozone dosage in the range 2-20 mg/L.

2. The decision to use ozone may be based on a need to reduce THM concentrations for public health reasons, but the selection of the applied dosage may be based on other factors such as destruction of micro-organisms, taste and odour control, colour removal and economic considerations.

3. As chloramine produced substantially lower THM and NPOX concentrations when applied as the secondary disinfectant, it would be the disinfectant of choice to provide a distribution system residual.

4. The differences in THM and NPOX production in different water supplies make pilot scale testing an important part of the selection of design criteria for the application of ozone to drinking water.

5. For water supplies containing high DOC (elevated THM), colour and bacterial/algal counts, "add-on" ozonation can deliver high quality treated drinking water at a 20-25% increase in cost to the consumer.

TABLE 1

Mean Chloroform Production by Hypochlorite-
Data Grouped by Ozone Dosage and Season

Nominal Applied Ozone Dosage	Mean Chloroform Production (ug/L) March (5°C)	July (26°C)	Sept. (13°C)
0(Pre NaOCl)	-	320±33.6(9)	319.6±40.1(8)
0	87.6±31.3(2)	253±38.8(6)	186.4±16.8(6)
2	54.1±31.3(2)	173±38.8(4)	132.0±18.5(4)
7.5	53.2±13.2(7)	175±27.9(7)	125.6±14.6(6)
20	39.2±7.4(4)	154±16.4(4)	105.3±29.8(4)

Note: Data presented as Mean ± Standard Deviation (No. of data points in mean)

TABLE 2

NPOX Concentrations: Data Grouped by Ozone Dosage and Season

Nominal Applied Ozone Dosage (mg/L)	Mean NPOX Production (ug/L) March[1]	July[2]	September[2]
0	152±80(2)	153	52
2.0	60±55(4)	88	20
7.5	100±33(7)	65	45
20	137±60(4)	112	35

[1] Data presented as Mean ± Standard Deviation (Number of data points in mean)

[2] Data are for analysis of random sample at each ozone dosage

TABLE 3

THM Production from DOC

	DOC (mg/L)	THM Production from DOC (ug/mg) Chlorinated	Ozonated/Chlorinated
a) Hypochlorite			
Brantford	6.1	28.5	18.3
Lindsay	10.5	15.4	11.4
Toronto	2.7	14.1	8.4
b) Chloramine			
Brantford	6.1	0.0	0.0
Lindsay	10.5	0.0	0.0
Toronto	2.7	0.0	0.0

TABLE 4

NPOX Production from DOC

	DOC (mg/L)	NPOX Production from DOC (ug/mg) Chlorinated	Ozonated/Chlorinated
a) Hypochlorite			
Brantford	6.1	54.2	21.0
Lindsay	10.5	24.9	18.8
Toronto	2.7	-	-
b) Chloramine		Chloraminated	Ozonated/Chloraminated
Brantford	6.1	10.7	7.4
Lindsay	10.5	10.8	6.5
Toronto	2.7	-	-

TABLE 5: COLIFORM REDUCTION IN PHASE 2
PRE-OZONATION

Nominal Applied Ozone Dosage (mg/L)	Mean Total Coliform Counts (per 100 ml) March		July		September	
	Raw	Ozonated	Raw	Ozonated	Raw	Ozonated
2.0	8	3	-	-	269	1
7.5	15	3	-	-	256	0
20	9	3	-	-	142	0

TABLE 6: TRUE COLOUR REMOVAL BY OZONATION

Nominal Applied Ozone Dosage (mg/L)	Mean Total Coliform Counts (per 100 mL)					
	March		July		September	
	Raw	Ozonated	Raw	Ozonated	Raw	Ozonated
2	20	5	30	L5	5	5
7.5	20	5	30	L5	5	5
20	20	5	30	L0	5	5

TABLE 7: TOTAL ANNUAL COSTS OF OZONATION SYSTEMS

Plant Size (m^3/day)	Ozone Dosage (mg/L)	Capital Cost ($1000)	Ann. Amort. Capital Cost ($/yr)	Ann. Oper. Cost ($/yr)	Total Ann. Cost ($/yr)	Unit Cost ($/$m^3$)
1,900	2.0	230	22,650	10,600	33,250	0.048
	7.5	285	28,000	14,700	42,700	0.062
19,000	2.0	615	60,150	22,000	82,150	0.012
	7.5	1210	118,500	54,700	173,150	0.025
190,000	2.0	3370	330,200	155,800	486,000	0.007
	7.5	6640	650,600	496,000	1,146,500	0.017

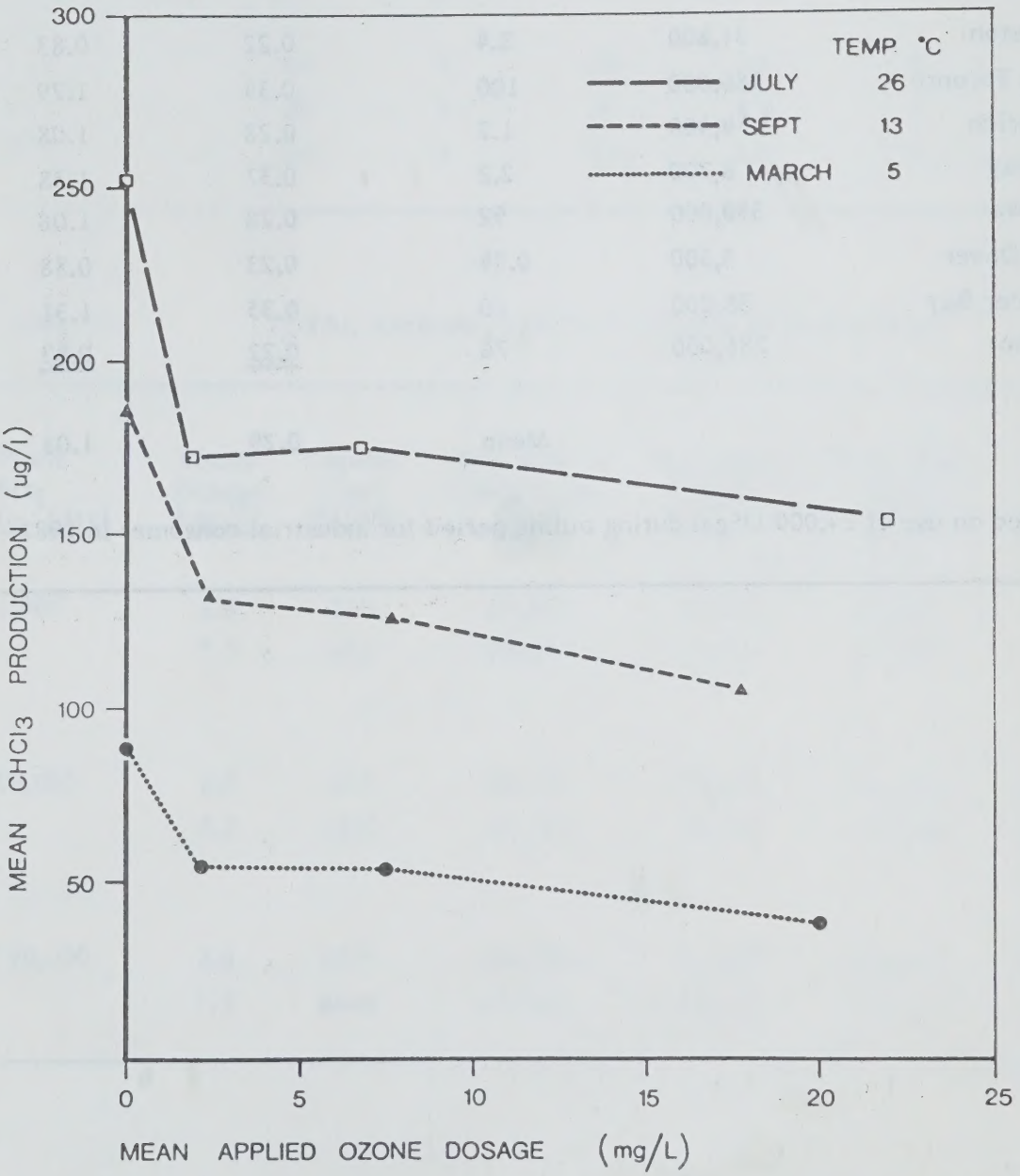
TABLE 8: TYPICAL COSTS OF MUNICIPAL WATER TO CONSUMER*

Municipality	Plant Size (m^3/day)	Plant Size (MUSGPD)	Cost ($/$m^3$)	Cost ($/1000 USgal)
Coniston	31,800	8.4	0.22	0.83
West Toronto	386,000	100	0.34	1.29
Goderich	4,400	1.2	0.28	1.08
Lindsay	8,200	2.2	0.37	1.38
Ottawa	350,000	92	0.28	1.06
Port Dover	3,500	0.94	0.23	0.88
Thunder Bay	38,200	10	0.35	1.31
Windsor	286,000	76	0.22	0.83
		Mean	0.29	1.08

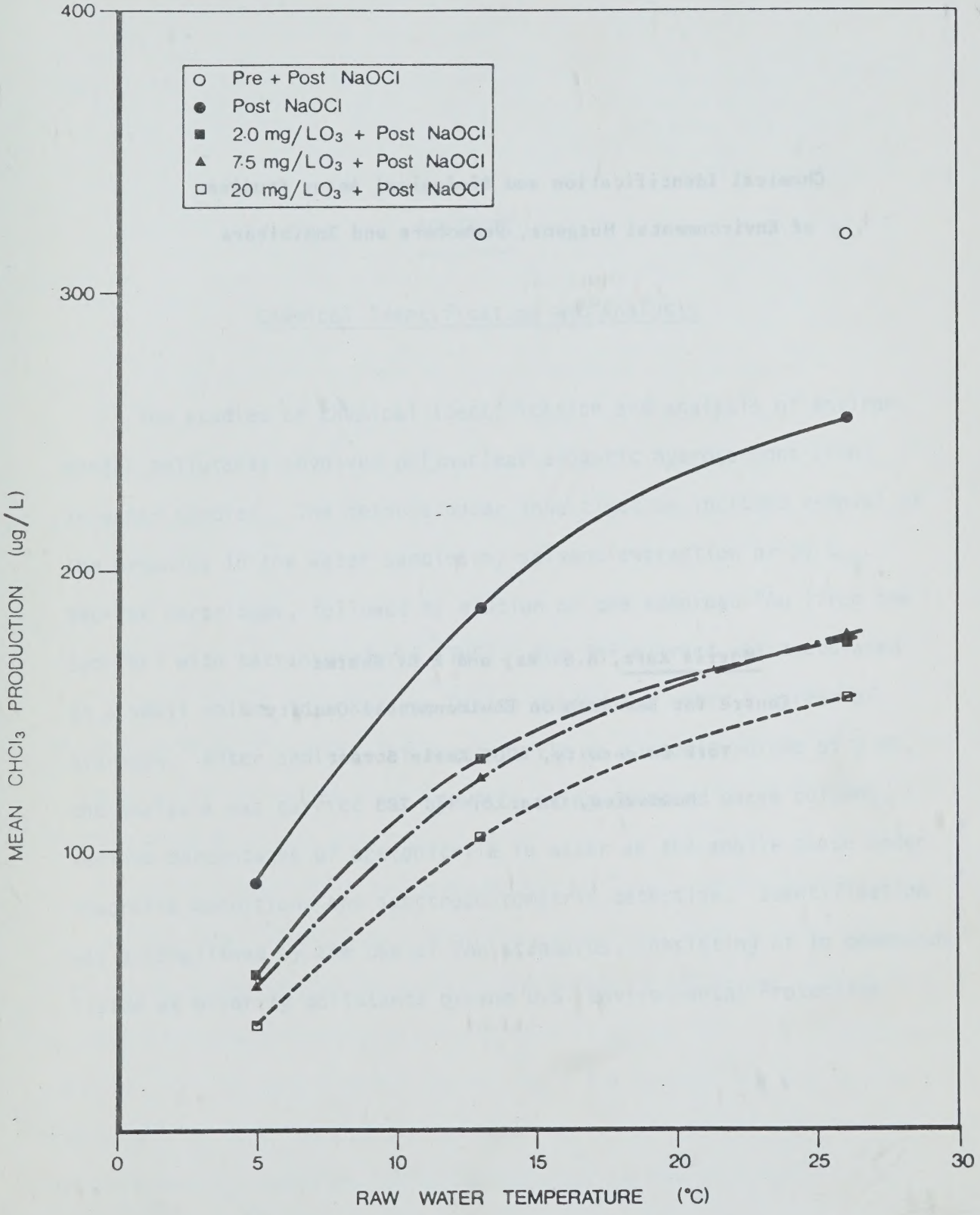
* Based on use of 24,000 USgal during billing period for industrial consumer in 1982

FIGURE 1

EFFECT OF PRE-OZONATION ON CHLOROFORM PRODUCTION IN POST-CHLORINATION



EFFECT OF SEASONAL CHANGES ON CHLOROFORM PRODUCTION



Chemical Identification and Biological Assay Studies of Environmental Mutgens, Promoters and Inhibitors

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ABSTRACT

Chemical Identification and Analysis

The studies of chemical identification and analysis of environmental pollutants involved polynuclear aromatic hydrocarbons (PAH) in water samples. The methods under investigation included removal of the organics in the water samples by solvent extraction or by C_{18} Sep-Pak cartridges, followed by elution of the adsorbed PAH (from the Sep-Pak) with tetrahydrofuran (THF). The THF extract was evaporated to a small volume in a Kuderna-Danish evaporator under a stream of nitrogen. After addition of acetonitrile to a sample volume of 2 ml, the analysis was carried out by HPLC, using reversed phase columns, various percentages of acetonitrile in water as the mobile phase under isocratic conditions and spectrophotometric detection. Identification was accomplished by the use of PAH standards, consisting of 16 compounds listed as priority pollutants by the U.S. Environmental Protection

Agency. Data recording was accomplished by a computing integrator. Mass spectra of PAH were also determined for identification purposes.

Water samples from the Welland River, collected at the steel bridges opposite the City of Welland and at Wellandport, were analyzed for eight PAH compounds. These were detected in much higher concentrations at Wellandport than in the samples at the City of Welland. The contaminants included the following carcinogens and mutagens:- benzo(a)pyrene, dibenz(a,c)anthracene, dibenz(a,h)anthracene, benzo(g,h,i) perylene and indeno(1,2,3-cd)pyrene.

A procedure has been developed to improve greatly the efficiency of extraction of PAH from water samples by C_{18} Sep-Pak cartridges involving the acidification of the water samples to pH 2 and prior activation of the Sep-Pak with THF. Recoveries of PAH ranged from over 80 to nearly 100 percent.

Biological Assay Studies

The biological studies of mutagens, potential innibitors and promoters have been conducted by means of a three-tier system of assays, consisting of the Ames Salmonella typhimurium test, and the *in vivo* bone marrow micronucleus and abnormal spermhead assays on mice. These assays have proved useful in delineating the mutagenic activity of representative PAH, such as 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene (BaP) and other chemicals, acting singly and in pairwise combinations. The mutagenic response of pairwise mixtures of promutagens such as BaP and DMBA or the other strong PAH mutagens was non-additive. However, the combination of a direct-acting mutagen such as mitomycin C (MMC) and BaP, yielded an additive response. Moreover, the combination

of the promutagens BaP and cyclophosphamide (CP) was found to be additive. CP induces a different enzyme system and metabolic pathway than that of BaP. Combinations of a direct-acting mutagen and a promutagen or of two promutagens that act by different enzymatic pathways (e.g., BaP and CP) were found to yield an additive clastogenic response in both somatic and germ cells.

A considerable number of complex phenols and antioxidants have been tested to determine their effectiveness as inhibitors of mutagenesis. Studies of inhibitors reported in the literature have been confined mainly to an assessment of their role in preventing or reducing neoplasia by carcinogenic agents. However, we have found that a number of such substances, e.g., butylated hydroxyanisole and cysteamine, which have been cited as effective inhibitors of chemical carcinogenesis, show no degree of inhibition whatever against the mutagens DMBA, BaP, CP and others, when tested by means of the in vivo bone marrow micronucleus assay. However, effective inhibition against the mutagenic effects of DMBA and BaP has been demonstrated by 7,8-benzoflavone, 5,6-benzoflavone, ascorbic acid, caffeic acid, ethoxyquin, disulfiram, α-tocopherol and β-sitosterol, corn oil and sunflower oil.

Preliminary studies have been conducted to determine the effects on PAH mutagens of promoters such as arochlor, phenobarbital and hexachlorobenzene, using the in vivo bone marrow micronucleus and abnormal spermhead assays.

I. CHEMICAL IDENTIFICATION AND ANALYSIS OF POLYNUCLEAR AROMATIC HYDROCARBONS IN WATER

The determination of polynuclear aromatic hydrocarbons (PHAs) and other organic compounds in water samples has usually been carried out by removal of organic material from the water using liquid-liquid extraction techniques (1,2,3,4,5), head space sampling techniques (6,7,8) or adsorption techniques employing solid adsorbents such as charcoal (9), Tenax (10), XAD-type resins (11) and polyurethane foam (12,13). Both XAD-4 aND XAD-8 in equal proportion were used in the column to extract organics of varying polarities from drinking water (14). More recently reversed-phase liquid chromatographic packing has been used to extract and concentrate trace levels of organics from water. May et al. (15) used a C_{18}-bonded phase packing for the extraction of hydrocarbons from water. Numerous reports have described the potential of reversed-phase packing for trace enrichment of organics from various aqueous samples (8,9,10,11,12). Traditional concentration and cleanup techniques employ multiple solvent extractions followed by solvent evaporation. These time consuming methods usually employ large amounts of solvents. There is, however, an urgent need for rapid methods because in many cases a large number of samples have to be analyzed. The technique of trace enrichment using a suitable column affords a simple means of simultaneous extraction, concentration and purification of trace amounts of organics from water samples in the same step. The present paper describes the feasibility of trace enrichment of water samples by commercially available Waters Sep-Pak C_{18} cartridges.

Concentrations of polynuclear aromatic hydrocarbons (PAHs) in drinking water sources and supplies have been found to range between a few nanograms to several hundred nanograms per liter (16,17,18). The techniques used to measure PAHs in environmental samples have been thin-layer chromatography, gas chromatography and GC-mass spectroscopy (19,20,21). However, each suffers certain inadequacies with respect to concentration, separation, detection or identification of PAHs. Separation of isomeric PAHs has been difficult with packed column GC analysis using both conventional and MS detectors (22,23). All these methods utilize liquid-liquid extaction, the traditional method of extracting and concentrating organic compounds from water which require several time consuming steps, large volumes of volatile organic solvents and considerable analytical skill. An alternative method is adsorption trapping, in which the organic compounds are extracted from a flowing sample by strong adsorption onto a solid matrix. Various adsorbents have been proposed as effective matrices for removal of organics from water (24,25,26).

More recently, reverse-phase octadecylsilane (ODS) chromatographic packing has also been used as a means of trace enrichment of organics from various aqueous samples including distilled water (27), drinking water (28,29), urine (30), chlorinated water (31), natural water (32) and waste water (33).

However, data on the efficiency of recovery and reproducibility of analytical methods based on adsorption trapping and trace enrichment of PAH vary widely with different solid matrices. It is advisable, therefore, to establish the isocratic conditions and extent of recovery of PAH for each particular type of solid adsorbent that is employed to trap such organics in water samples.

Instrumentation

Chromatography was conducted using Waters liquid chromatograph Model 204 with dual Model 6000A solvent delivery pumps, a model U6K universal injector, a Model 600 solvent programmer, Supelco LC-PAH reversed-phase analytic column and Waters radial compression separation system containing Z-module and a Radial-Pak PAH analytical cartridge column. Data recording was done on a Spectra Physics Model 4270 Computing Integrator. Detection was accomplished via Waters Model 440 UV detector at 254 nm.

Preparation of Standards and Samples

The PAH standards used in this study were obtained from Supelco containing 16 PAHs which comprise the U.S. Environmental Protection Agency (EPA) list of priority pollutants for water analysis. These compounds were obtained as solutions in methanol/dichlormethane 50/50 (V/V) and were diluted further, 1 ml to either 10 ml or 100 ml, in acetonitrile prior to use as standards. The stock solutions of standard PAHs were stored in a refrigerator. The synthetic samples of water were prepared by adding 1 ml of diluted standard PAH solution to 50 ml of high purity water from Millipores-Milli Q purification system.

Procedure

Each Waters Sep-Pak cartridge contains 0.35g of C_{18} porous (70 μm particle size) packing material (140 m^2 of surface area per cartridge). The Sep-Pak obviated hand-packing of non-disposable guard columns and thus reduced any accidental contamination and packing variations associated with this procedure. More importantly, Sep-Pak cartridges allowed the use of hand-held syringes instead of electrical pumps to pass through water samples for extraction of organics, thus improving portability for on-site sampling. These cartridges are made specifically to fit a Luer-lock tipped syringe for introduction of solution or solvents manually.

Prior to trace enrichment, the Sep-Pak cartridge was always activated by passing 10ml tetrahydrofuran, 10ml methyl alcohol followed by a rinse of water at pH 2. Then 50ml of synthetic water samples (1ml of PAH standard in 50ml of water) at pH 2 was passed through an activated Sep-Pak at a flow rate of 1 drop/sec. The Sep-Pak was dried by passing air through it until no water drops were observed. The adsorbed PAHs were eluted with 10ml of glass-distilled dichloromethane into a 25ml pear-shaped flask. Dichloromethane was removed completely from the extract with a stream of dry nitrogen by a rotary evaporator. One ml of glass-distilled acetonitrile was then added to the residue and 10 or 25µl of this sample was analysed by HPLC.

Runs were carried out under both isocratic and gradient conditions. Quantitation was accomplished by comparing the sample peak areas to those of standards obtained under identical conditions.

Results and Discussion

Figure 1 shows the chromatogram of 16 PAH standards obtained from Waters Z-module consisting of a Radial Pak PAH cartridge column. Table 1 lists the peak number of the PAHs in order of increasing elution time and their concentrations. Figure 2 shows the chromatogram of PAHs concentrated from synthetic water samples at pH 2 by Sep-Pak.

It was found that the extraction of PAHs from the synthetic water samples by Sep-Pak, without pH adjustment of the water, yielded a wide range of percent recovery. A similar trend was observed in the liquid-liquid extraction of the PAHs with dichloromethane from water samples at about pH 7.

Table 2 shows the percent recovery of PAHs obtained from the synthetic

TABLE 1: Retention Time of PAHs (Supelco Standard) and Their Concentration

	PAH Compounds (in order of elution)		Retention (min)	Concentration (mg/ml)
1	Naphthalene	Nph	4.49	0.10
2	Acenaphthylene	Acelene	6.06	0.20
3	Acenaphthene	Ace	8.75	0.10
4	Fluorene	Fl	10.14	0.02
5	Phenanthrene	Phe	13.44	0.01
6	Anthracene	An	16.04	0.01
7	Fluoranthene	Ft	18.16	0.02
8	Pyrene	Py	19.12	0.01
9	Benz(a)anthracene	B(a)An	23.57	0.01
10	Chrysene	Chy	24.18	0.01
11	Benzo(b)fluoranthene	B(b)Ft	27.26	0.02
12	Benzo(k)fluoranthene	B(k)Ft	28.70	0.01
13	Benzo(a)pyrene	B(a)Py	29.60	0.01
14	Dibenz(a,h)anthracene	diB(a,h)An	32.14	0.02
15	Benzo(g,h,i)perylene	B(g,h,i)Per	32.64	0.02
16	Indeno(1,2,3-cd)pyrene	In(1,2,3-cd)Py	33.71	0.01

water samples when the pH was not adjusted to 2. It was found that the high molecular weight PAHs such as diB(ah)An, B(ghi)Per and In(1,2,3-cd)Py gave a low recovery by both Sep-Pak and liquid-liquid extractions, without pH adjustment. Several runs were carried out to investigate the recovery of B(ghi)Per in 50 ml of water (without pH adjustment) by both Sep-Pak and liquid-liquid extraction using dichloromethane. Similar experiments were performed with this PAH in water samples adjusted to pH 2 and the results indicated greatly improved recovery (see Table 2, footnote).

Clearly, both liquid-liquid extraction and Sep-Pak enrichment gave higher recovery percentages from synthetic water samples at pH 2 as shown in Tables 2 and 3. Similar observations were made by Eichelberger et al. (34) in their study of the recovery of sixteen PAH compounds on the EPA priority list when extracted from water samples adjusted to pH 2. However, they reported mean relative standard deviations in percent recovery of PAHs for packed column and capillary column methods of 20%, whereas our results for the percent recovery of 16 PAHs by Sep-Pak from synthetic water samples at pH 2 indicate a relative standard deviation of less than 10%. Percent recovery, as shown in Table 3, of all PAHs except naphthalene, is in the range of 80 to 94%.

Polycyclic Aromatic Hydrocarbons (PAH) in Welland River

The lower Welland River is the recipient of domestic effluents from the City of Welland and of liquid wastes from many industrial plants. Industries include steel plants, textile mills, abrasive plants, a large chemical plant and various support industries. All of these operations contribute to the load of effluents carried into the lower Welland River.

TABLE 2: Percent Recovery of PAHs from Synthetic Water Samples Without pH Adjustment

	% Recovery Liquid-Liquid Extraction		% Recovery Sep-Pak Extraction		
Nph	39	44	0	0	20
Acelene	62	33	28	51	60
Ace	60	21	46	65	74
Fl	70	29	0	78	72
Phe	80	83	0	106	-
An	79	55	65	89	79
Ft	78	14	81	105	43
Py	95	80	63	108	59
B(a)An	77	-	56	85	25
Chy	78	-	41	77	20
B(b)Ft	77	32	58	71	47
B(k)Ft	72	79	44	64	31
B(a)Py	69	73	46	70	37
diB(a,h)An	72	57	0	30	0
B(g,h,i)Per	72	56	23	34	19
In(1,2,3-cd)Py	76	59	18	67	31

Recovery of B(g,h,i)Per from synthetic water samples without pH adjustment:
Liquid-liquid extraction gave 66, 55 and 60% recovery.
Sep-Pak extraction gave 25, 63 and 55% recovery.
Same experiments with pH adjusted to 2:
% Recovery of B(g,h,i)Per by liquid-liquid extraction - 95.5% (average of 4 runs)
-by Sep-Pak extraction - 89.8% (average of 4 runs).

TABLE 3: Recovery of 16 PAHs from Synthetic Water Samples at pH 2

PAH	Sep-Pak Extraction Percent Recovery (average of 5 runs)	Relative Standard Deviation (%)
Nph	13.2	-
Acelene	82.9	9.7
Ace	79.7	5.6
Fl	83.5	6.1
Phe	87.5	7.7
An	86.8	7.6
Ft	91.3	7.0
Py	84.8	9.3
B(a)An	89.4	5.8
Chy	91.3	9.0
B(b)Ft	92.7	6.7
B(k)Ft	92.0	5.6
B(a)Py	92.5	6.2
diB(a,h)An	93.9	4.6
B(g,h,i)Per	93.8	5.9
In(12,2,3-cd)Py	94.4	6.3

FIGURE 1: Chromatogram of Supelco Standard (10 x diluted) Consisting of 16 PAHs.

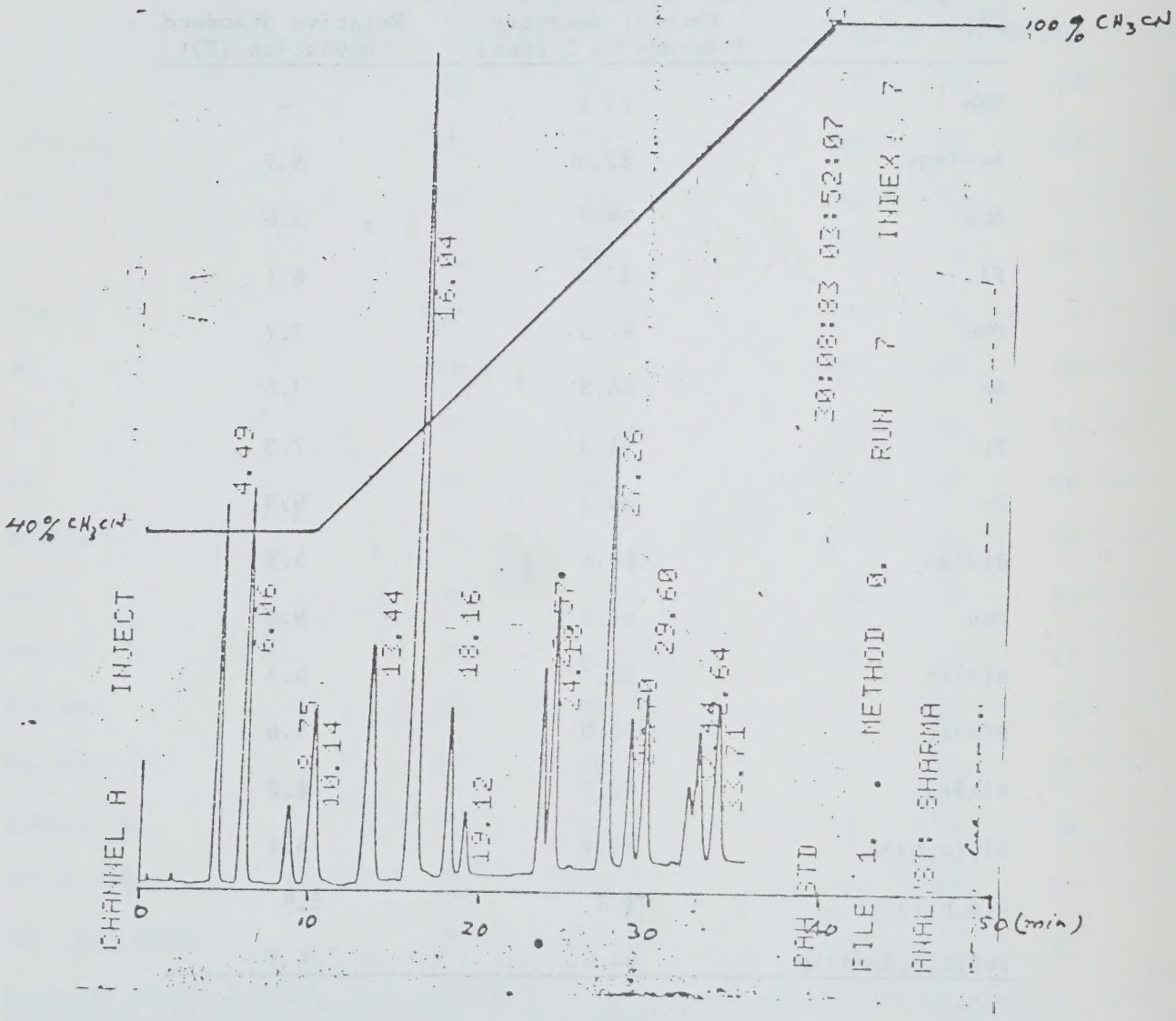
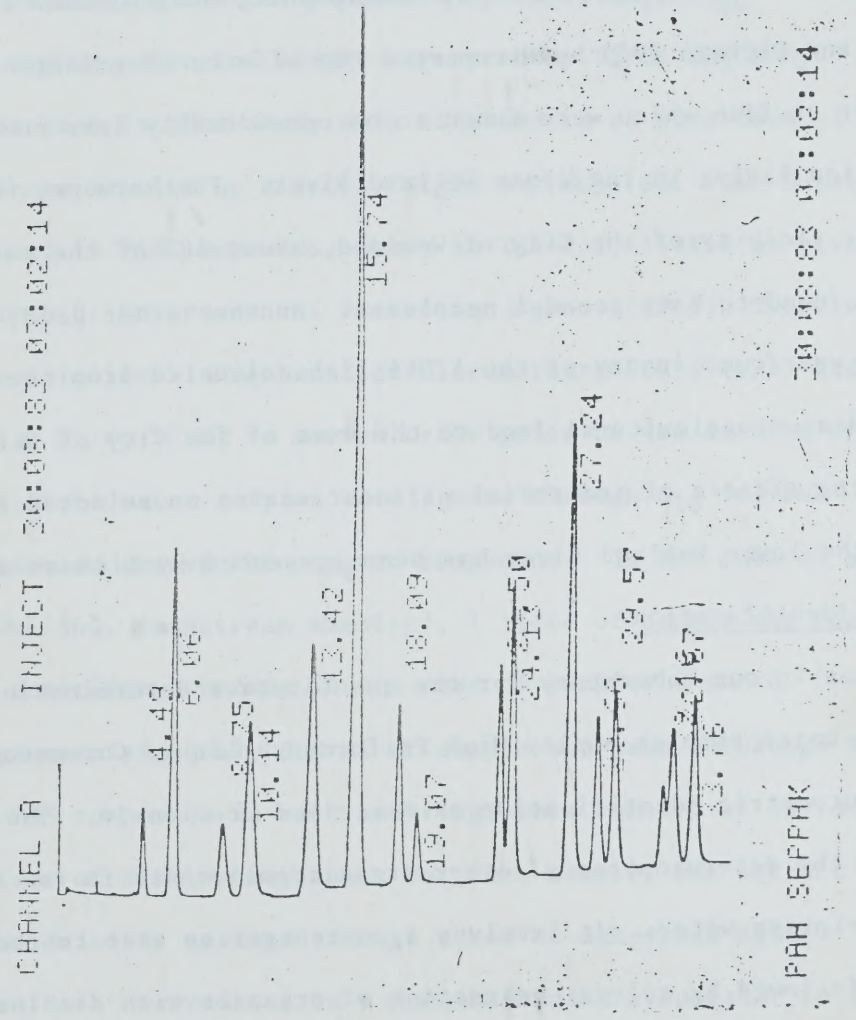


FIGURE 2: Chromatogram of 16 PAHs Extracted by Sep-Pak from Synthetic Water Sample



In order to determine the presence and concentration of PAH compounds in this river, samples of water were obtained through the cooperation of Professor M. Dickman, Brock University,, St. Catherines, who supplied four 1-litre samples on May 25, 1982. Samples 1 and 2 were taken at the West Main and Prince Charles steel bridges in the City of Welland (specified as downstream samples). Upstream samples 3 and 4 were taken at Wellandport, near Gladman's Park.

Steele and Dickman (35) have reported the effects of pollution of the lower Welland River on fish which were found to be considerably less numerous than the fish population living in the upper Welland River. Furthermore, in the lower river, in the vicinity of the City of Welland, about 48% of the carp-goldfish hybrids were found to have gonadal neoplasms. On ther other hand, no evidence of neoplasms was found in any of the 1,066 fish collected from the upper Welland River which drains agricultural land to the west of the City of Welland. Other evidence of the effects of industrial effluent wastes on selected aquatic plants and fish in the lower Welland River has been presented by Dickman et al. (36).

Analysis and Identification

Techniques in our laboratory for the quantitative determination of PAH pollutants in water samples employ High Performance Liquid Chromatography (HPLC) and spectrophotometric identification as described previously. The method is applicable to the determination of other organic pollutants in raw water and chlorinated drinking water. It involves a concentration step to reduce the sample size, followed by solvent extraction of organics with dichloromethane, evaporation to a small volume at room temperature and subsequent HPLC analysis, using reversed-phase columns and isocratic conditons, with mixtures of acetonitrile-water as the mobile phase.

Trace concentrations of PAHs can be separated into subgroups as follows, under isocratic conditions:

a) Two and three membered ring systems separated by 60/40 and 50/50 CH_3CN/H_2O

b) Four membered ring systems separated by 75/25 CH_3CN/H_2O

c) Five membered ring systems separated by 85/15 CH_3CN/H_2O

d) PAHs of higher molecular weight separated by 95/05 CH_3CN/H_2O

The PAH standards used in this study were obtained in as pure form as possible from various sources, including Aldrich, Sigma and Supelco. The Supelco standards contained 16 PAH compounds, shown in Table 1, listed as priority pollutants by the U.S. Environmental Protection Agency (EPA). These compounds were obtained as solutions in methanol/dichloromethane 50/50 (v/v) and were diluted further, 1 ml to 10 ml in acetonitrile prior to use as HPLC standards.

The Welland River water samples were passed through C_{18} Sep-Pak cartridges in order to remove the organic compounds. In the case of samples 1 and 2 (City of Welland, downstream samples), 1 litre of sample was passed through the C_{18} cartridge, whereas in the case of samples 3 and 4 (Wellandport, upstream samples), 500 ml of sample was passed through the C_{18} cartridge. The PAH compounds in each cartridge were removed by extraction with successive portions of dichloromethane. For each sample, the extract fractions were combined and dried by passage through a bed of anhydrous $MgSO_4$. The volume of each extract was reduced in a Kuderna-Danish evaporator to about 5 ml, and the remaining dichloromethane was removed by evaporation at room

temperature under a stream of nitrogen. Then 0.5 ml of acetonitrile was added to the organic residue.

The solvent program for HPLC analysis, using various percentages of acetonitrile in water, included a reversed-phase column and ultraviolet detection at 254 nm. The PAH compounds of high molecular weight, containing 5 and 6 condensed aromatic ring systems are separated by solvents containing 85% and 95% acetonitrile in water. Lower concentrations of acenotnitrile in water must be employed to separate compounds consisting of 2 to 4 aromatic rings.

The HPLC separation of PAH from the Welland River samples are illustrated in Figure 3 for upstream samples 3 and 4, and in Figure 4 for the downstream samples 1 and 2, on the basis of µl injections of concentrated PAH extracts. The numbers on the various peaks in each chromatogram identify PAH compounds listed in the standards.

The concentrations, shown in Table 4, were determined by first evaluating the response factors (RF), defined as the ratio of the peak area to the amount of analyte standard injected. Thus, if analysis of a sample extract yields X ng injected of a given PAH (peak area times response factor), the concentration of this compounds in the original water samples is given by the formula

$$C = X / (rV) \quad ng/l$$

where C = concentration, r is the ratio of the injection volume to the total extract volume and V is the volume in litres of the original water sample. The extraction efficiency for each PAH has been taken at 100%.

The results in Table 4 indicate that the concentrations of the eight listed PAH compounds are considerably higher in the water taken upstream at Wellandport

than the concentrations in the water collected under the steel bridge at the City of Welland.

Carcinogenic and Mutagenic Activity

The carcinogenic and mutagenic activities of the eight PAH compounds found in the Welland River water samples are listed in Table 5. Dipple (37) has reviewed extensively the findings of many investigators' *in vitro* and *in vivo* studies of polynuclear aromatic carcinogens and the information in Table 5 on carcinogenic activity is based on data listed in this review.

The remarks regarding findings of mutagenic activity of the PAH (Table 5) are based on tests using the Ames assay as published by Salamone, Heddle and Katz (38). In general, the various results based on the Ames assay agree with the reported carcinogenic potency of these PAH.

The results of studies of some PAH concentration levels (ng/l) in several U.S. river waters are listed in Table 6 for purposes of comparison with values obtained in the Welland River samples (Table 4). The comparatively high values quoted by Basu and Saxena (39) were found in water collected in the Monogahela River, near Pittsburg, contaminated with coke oven effluent.

The analysis of the above water samples from the Welland River was undertaken prior to the study of the efficiency of Sep-Pak cartridges for the quantitative removal of PAHs in water. Since it has been revealed that requirements for quantitative recovery of dissolved PAH involves an activation procedure for the Sep-Pak and acidification of the water sample to pH 2, the above PAH concentrations in the Welland River samples must be accepted with reserve, as the data are unsupported by evidence of recovery efficiency.

TABLE 4: PAHs in Welland River Samples

	Up stream (ng/l)	Down stream (ng/l)
Chrysene	7.3	2.5
Benzo(e)pyrene	40	6.4
Perylene	21	5.0
Dibenz(a,c)anthracene	68	10
Benzo(a)pyrene	17	4.6
Dibenz(a,h)anthracene	72	24
Benzo(ghi)perylene	129	33
Indeno(1,2,3-cd)pyrene	2.0	0.5

TABLE 5: Carcinogenic and Mutagenic Activity of PAH in Welland River Samples

PAH Compound	Carcinogenic activity (Dipple)	Mutagenic activity (Salamone, Heddle & Katz)
Chrysene	disputed	slight
Benzo(e)pyrene	inactive	doubtful
Benzo(a)pyrene	high	high
Perylene	inactive	inactive
Dibenz(a,c)anthracene	disputed	mild
Dibenz(a,h)anthracene	moderate	mild
Benzo(ghi)perylene	moderate	mild
Indeno(1,2,3-cd)pyrene	moderate	-

TABLE 6: PAH Concentration Levels in Some U.S. River Waters (concentrations in ng/l)

PAH Compound	Crane et al. (40)	Organ et al.(41)	Basu and Saxena (39)
Chrysene	-	70	-
Benzo(a)pyrene	17	6	42
Benzo(ghi)perylene	19	16	34
Indeno(1,2,3-cd)pyrene	10	10	60

FIGURE 3.

SOLVENT: 85% ACETONITRILE

SAMPLE: UPSTREAM SAMPLE

FROM WELLAND RIVER

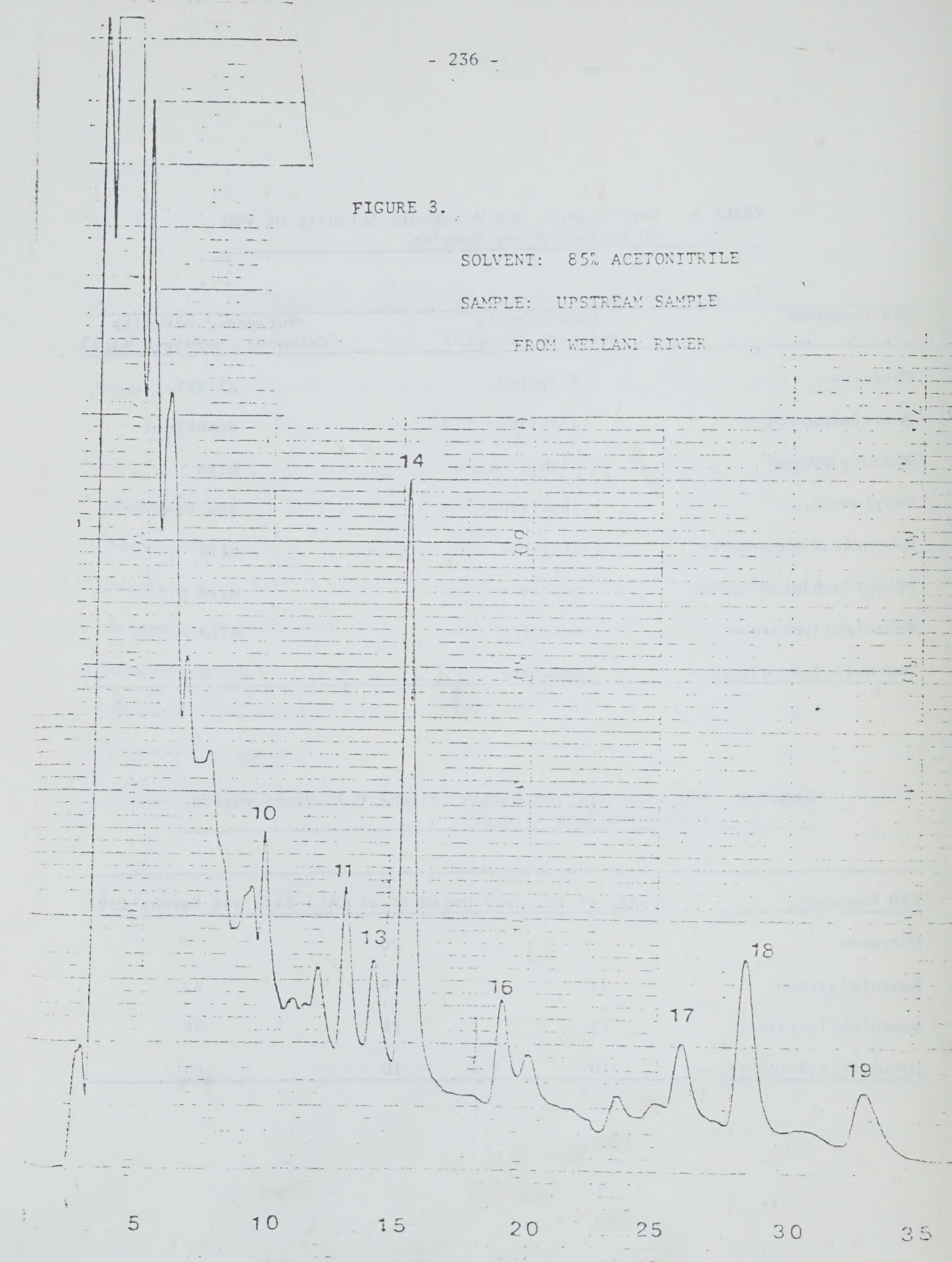
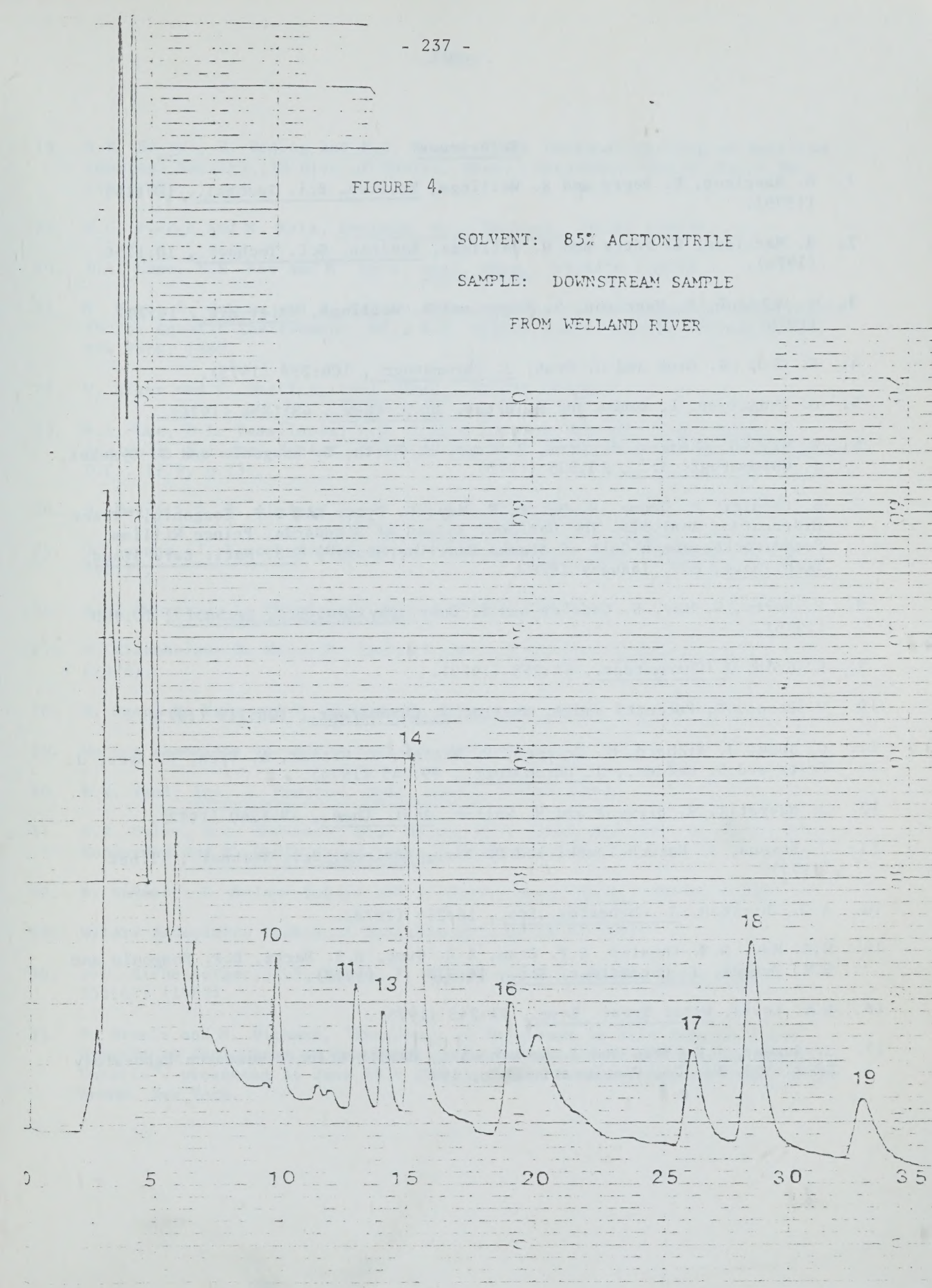


FIGURE 4.

SOLVENT: 85% ACETONITRILE

SAMPLE: DOWNSTREAM SAMPLE

FROM WELLAND RIVER



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II. BIOLOGICAL STUDIES OF ENVIRONMENTAL MUTAGENS, INHIBITORS AND PROMOTERS

Introduction

It has been estimated that 60% or more of the incidence of cancers in man and animals is due to the action of environmental chemicals or pollutants. Anthropogenic sources of carcinogenic and mutagenic chemicals include the products of incomplete combustion of fossil fuels, exhaust gases from motor vehicles, emissions from coke ovens and from many other industrial activities, such as oil refining, chemical manufacturing and metal processing. Polycyclic aromatic hydrocarbons (PAH) are a particularly important group of carcinogenic pollutants but there are many other classes of environmental substances that constitute potential carcinogenic or mutagenic hazards to health, including chlorinated and fluorinated hydrocarbons, alkylating agents, amines and nitrosamines, and other organic compounds and inorganic chemicals containing Be, Cd, Mn, Ni, Co, Pb and Cr.

Extensive reviews of the activity of many chemical carcinogens have been published by Heidelberger (1), Searle (2), the Millers (3), the U.S. National Academy of Sciences (4) and others (5,6,7). Apart from anthropogenic sources, a considerable number of carcinogens are products of natural origin and occur in plant species and micro-organisms, e.g., alkaloids, constituents of essential oils, aflatoxins. The anthropogenic chemicals, such as PAH, are prevalent contaminants of air, water and soil.

Various theories have been proposed to explain the mechanism of action of chemicals in terms of species and tissue susceptibility, biochemical pathways for activation and detoxification and elucidation of the role of specific

classes of compounds in human carcinogenesis. An extensive review of the chemistry and biological effects of PAH has been presented in the proceedings of a symposium edited by Bjorseth and Dennis (8). These compounds can bind covalently to cellular macromolecules such as DNA, RNA and protein. The amount of hydrocarbon bound is proportional to the concentration of macromolecule in the tissue, with most of the hydrocarbon being found by cellular protein, followed by RNA and DNA. Since PAH are metabolized by a microsomal mixed-function oxidase enzyme called aryl hydrocarbon hydroxylase (AHH), the resultant activation process may lead to the formation of an intermediate epoxide species which is a much more active carcinogen than the parent hydrocarbon. The epoxide can be converted into a glycol or diol by a second microsomal enzyme, epoxide hydrase, which may lead to detoxification and rapid removal of the reactive epoxide before interaction with a target site (9). However, in some cases the diols of PAH can undergo another cycle of metabolic activation and become even more carcinogenic, as diol epoxides, than the initial epoxide (10). The diol epoxide of benzo(a)pyrene (BaP) has been shown to be an active mutagen (11).

Aromatic amines and nitrosamines also require metabolic oxidation by hepatic microsomal mixed-function oxidases to become tumorigenic (9). Carcinogenic substances that do not require metabolic activation are biological alkylating agents, such as alkyl alkanes, epoxides, sulfonates, strained ring lactones, and nitrosamides. Inorganic carcinogenic chemicals containing certain toxic metals and metalloids, and asbestos, are also effective without metabolic activation.

A review of the etiology, mechanisms and prevention of cancer has been presented by Slaga (12). Chemical carcinogenesis is a multistep process as demonstrated by studies with experimental animals. In addition to the action of tumor initiators and promoters, other factors include the effect of the presence of other carcinogens acting to produce additive, synergistic or inhibitory effects. Agents are also known that can act to inhibit the process by modifying metabolic pathways to counteract or detoxify the carcinogen. The DNA repair mechanism is an important determining factor in the cancer initiation of a cell by a chemical carcinogen or mutagen. Interference or inhibition of the DNA excision repair system may lead to the irreversible carcinogenic process.

Some of the known cancer promoters include croton oil and its phorbol esters, fatty acids and esters, tobacco smoke condensate, phenols, DDT and PCB. Various chemical agents have been studied that are potential inhibitors of both the initiation and promotion phases of carcinogenesis. These substances have the capacity to inhibit tumor initiation or promotion by one or more of the following pathways (13,14): (a) alteration of the metabolism of the carcinogen by microsomal enzymes, leading to decreased activation and/or increased detoxification, (b) scavenging of active carcinogens to prevent their approach to critical target sites in the cells, (c) inhibition of promotion or progression by altering the state of differentiation, (d) inhibition of promoter-induced cellular differentiation, (e) prevention of gene activation by promoters. Inhibitors that have been investigated include flavones, proteases, retinoids, anti-inflammatory steroids, and a number of antioxidants. Cruciferous plants and citrus fruits contain certain chemicals that act as

inhibitors and inducers of the mixed-function microsomal oxidase system and result in enhanced detoxification of carcinogens (15).

Within recent years, the concern regarding the continued increase in the numbers of chemical carcinogens in the environment has led to greatly increased efforts to survey, identify and study the chemistry and biological effects of potential carcinogens. However, conventional tests with animals, especially for unknown or weak carcinogenic activity, require costly studies for long periods. Current standards for testing carcinogens in mice involve a cost of at least $150,000 for a single chemical. Such cost and the two-year time period required for each test render the conventional methods completely unsuitable for experiments involving a large number of chemicals and combinations of chemicals. Typical human exposure to air pollutants involves not merely single compounds but rather complex mixtures of large numbers of PAH and other chemicals.

Much more rapid and economical testing methods have been developed in recent years. These new methods for detecting carcinogens depend upon the ability of potential carcinogens to produce mutations or genetic damage in bacterial and mammalian cells. Both in vitro and in vivo biological assays are now available to detect agents that can produce mutations.

The following comparatively rapid and sensitive assays for mutagenic activity of chemicals have been employed in this study.

1. The Ames Salmonella typhimurium assay

This assay, developed by Ames and his co-workers (16,17), involves the use of histidine auxotrophs of the above bacteria, with strains TA1535 and TA100 each bearing a substitution-deletion mutation or TA1538, TA98 and TA1537 each

containing a frame shift mutation. Results by McCann et al. (17) of 300 chemicals containing all known human carcinogens, except the heavy metals, have indicated that on the above strains 85% of the carcinogens are also mutagens.

2. In vivo mammalian assays

(a) A micronucleus assay, developed by Heddle (18), Schmidt (29) and Salamone et al. (23) detects damage to the chromosomes of the rapidly dividing erythroblastic cells in mice bone marrow. The test is based on the phenomenon that chromosome fragments which lack spindle attachment sites or centromeres are often left in the cytoplasm of dividing cells where they persist as micronuclei. Such structures are readily recognized and can be scored rapidly compared to the classical methods of metaphase chromosome analysis.

(b) The sperm abnormality assay detects point mutations leading to abnormal sperm head morphology as a result of exposure of male mice to irradiation and chemicals that are mutagens, teratogens and carcinogens (19,20,21).

Earlier studies in the field, published by our research group, have dealt with the mutagenic activity of 30 PAH and oxides in airborne particulates (22), development of an improved micronucleus test (23), mutagenicity of paired chemical mixtures (24), the mutagenic activity of 41 compounds on the in vivo micronucleus assay, as part of an international collaborative program (25), mutagenic activity of PAH and other environmental pollutants (26), the complexities of risk estimates, metabolic activation and chemical mixtures (27) and the effect of 5,6-benzoflavone on the mutagenicity of PAH (28).

Various chemicals which have been used in the above tests are listed below with their sources. Methods of operation have been described in the individual assays.

TABLE 1

Chemicals	Source	Mutagen/inhibitor/promotor
Dimethylbenz(a)anthracene (DMBA)	Eastman Kodak Co.	Promutagen
Benzo(a)pyrene (BaP)	Aldrich Chemical Co.	Promutagen
Cyclophosphamide (CP)	Aldrich Chemical Co.	Promutagen
Mitomycin C (MMC)	Boehringer Mannheim	Direct acting mutagen
Butylated hydroxyanisole (BHA)	Sigma Chemical Co	Possible inhibitor
α-naphthoflavone	Sigma Chemical Co.	Possible inhibitor
β-naphthoflavone	Sigma Chemical Co.	Possible inhibitor
Caffeic acid	Fluka Co.	Possible inhibitor
Ascorbic acid	BDH Chemical Co.	Possible inhibitor
Disulfiram	Sigma Chemical Co.	Possible inhitibor
α-tocopherol	Hoffman La Roche	Possible inhibitor
β-sitosterol	Sigma Chemical Co.	Possible inhibitor
Ethoxyquin	Aldrich Chemical Co.	Possible inhibitor
Corn oil	Mazola Brand/retail store	Possible inhibitor
Sunflower oil	Unico Brand/retail store	Possible inhibitor
Metyrapone	Aldrich Chemical Co.	Possible inhibitor
Arochlor	Sigma Chemical Co.	Promoter
Phenobarbital	Sigma Chemical Co.	Promoter
Hexachlorobenzene	Aldrich Chemical Co.	Promoter
Hexachlorocyclohexane	Aldrich Chemical Co.	Promoter

I. Bone Marrow Micronucleus Assay

Virgin B6C3 female mice (Charles River Canada Inc.) were 8-9 weeks of age when used for *in vivo* bone marrow micronucleus assay experiments. The mice ranged from 20-22g in weight and they were maintained under normal animal room conditions before and during experimental periods. All mice were fed with Purina Laboratory Rodent Chow and watered ad libitum. Five animals were housed per cage (cage dimensions, 19cm W x 29cm L x 12 cm H) and 5 animals were used per each treatment. The *in vivo* bone marrow micronucleus assay as described by Schmidt (29) and Salamone et al. (23) has been employed in the present studies. $LD_{50/7}$ was determined for the chemicals before being tested (Weil (30)).

The mice were injected intraperitoneally (i.p.) with the chemical dissolved in appropriate solvent and samples of bone marrow taken at various intervals starting from 24 hr after the treatment with test compounds. Micronucleated polychromatic erythrocytes (MNPCE) per 500 PCE were scored in each sample. Control animals were treated with solvent only and tested for the presence of micronuclei as above. Red blood cells (RBC) have also been counted per 100 PCE. The ratio of RBC/PCE gives the approximate estimate of toxicity of the test chemical.

In general, dimethyl sulfoxide (DMSO) has been used as a solvent. However, distilled water, physiological saline, and corn oil also have been used as appropriate solvents in some cases.

Potential inhibitors were injected 48 hr and 24 hr before treating the mice with promutagen or direct acting mutagen. By giving two injections 24 hr apart, one can give double the amount of chemical to the same mouse. So by injecting the presumed inhibitor 48 hr before injecting the mutagen, this probably facilitates building up of the aryl hydrocarbon hydroxylase enzyme system so

that this enzyme complex can detoxify the effect of the promutagen. The mice were treated with the potential inhibitors listed in Table 1.

Some of the inhibitors were mixed in powdered food and the experimental mice were kept on that diet for at least 7 days before treating with the mutagen. Butylated hydroxyanisole (BHA), caffeic acid, disulfiram, ethoxyquin were the inhibitors fed in this way to the mice.

Potential Inhibitors of Mutagens - In Vivo Bone Marrow Micronucleus Assay

1. Butylated Hydroxyanisole (BHA)

Experimental mice were fed with powdered food containing BHA (5g/Kg of food) for one week before administering mutagens and continued feeding until the end of the experiment. Then those mice were injected i.p. either eith DMBA, BaP, CP or MMC. Bone marrow samples were collected at various intervals up to 96 hr starting from 24 hr after administering the mutagen. Our results showed that at 56 hr sampling time there was about 133% increase in the mean number of micronucleated polychromatic erythrocytes in BHA + DMBA treatment over DMBA treatment alone (Fig. 1). So, BHA showed a large promotory effect in the presence of DMBA. Our result was quite contradictory to the results of Wattenberg (14,15) where he indicated that BHA had inhibited the carcinogen-induced neoplasia either in lung or forestomach or both in mice.

Neither promotory nor inhibitory effect was noticed with BHA + BaP, BHA + CP or BHA + MMC.

2. 5,6 Benzoflavone or β-naphthoflavone

Mice wre pretreated i.p. with β-naphthoflavone (80mg/Kg in DMSO) for two days consecutively before treating with either DMBA (25mg/Kg in DMSO) or BaP (186mg/Kg in DMSO). In another set β-naphthoflavone, BaP and DMBA were

dissolved in corn oil. In the former case, β-naphthoflavone dissolved in DMSO showed enhanced activity of DMBA instead of the expected reduction. However, in the latter case, where corn oil was used as a solvent for β-naphthoflavone as well as DMBA, the number of micronucleated PCE was reduced by 50%. Therefore, the actual inhibition was suspected to be due to corn oil.

β-Naphthoflavone in DMSO acted as an inhibitor by reducing the number of micronucleated PCE by 60% in the experiments where BaP was used as the promutagen. But, 100% inhibitory effect was noticed where β-naphthoflavone and BaP were dissolved in corn oil. Here also, corn oil could have actually enhanced the inhibition.

3. Alpha Naphthoflavone (7,8-benzoflavone)

In pulmonary adenoma test system of the mouse, flavone inducers inhibited lung tumor formation resulting from oral administration of DMBA or BaP (14,15). In other studies (5), it was reported that 7,8-benzoflavone inhibits hydrocarbon metabolism and hydrocarbon toxicity in hamster cell homogenates and rat liver microsomes only when these activities have been induced by prior treatments with benz(a)anthracene and 3-methylcholanthrene respectively. It has also been observed that 7,8-benzoflavone acted as a potent inhibitor of microsomal mixed function oxidase activity resulting ultimately in inhibition of epidermal neoplasia caused by DMBA (7,13).

The above information substantiates the inhibitory action of 7,8-benzoflavone in various types of cancer. Consequently we examined whether 7,8-benzoflavone would inhibit the formation of chromosomal breaks in mouse bone marrow tissue induced by DMBA or BaP.

Mice were injected, i.p., with 7,8-benzoflavone (80mg/Kg in DMSO) at 48 and 24 hr (2 injections per mouse) before treating with either DMBA (25mg/Kg in DMSO) or BaP (150mg/Kg in DMSO). Bone marrow samples were taken at prescribed times (24, 48, 72 and 96 hr after treatment with either DMBA or BaP).

Bone marrow cells from mice treated with 7,8-benzoflavone alone showed less than one micronucleated PCE per 500 PCE on average, as found also with DMSO. Maximum number of micronucleated PCE observed on average was 8.6 at 48 hr with DMBA and 11.6 at 72 hr with BaP. Considerable reduction in the average number of micronucleated PCE in the above treatments was found when the mice were pretreated with 7,8-benzoflavone. In other words, about 90% inhibition in case of DMBA and 75% in case of BaP was caused by 7,8-benzoflavone. Detailed results have been published elsewhere (Raj and Katz (31)).

4. Caffeic Acid

Caffeic acid (3,4-dihydroxycinnamic acid) is present in the free state only in small quantities in foods of vegetable origin but constitutes a significant portion in polymeric form of the lignin fibres that hold wood plant cells together. Caffeic acid is present in potatoes, sweet potatoes, apples, pears, carrots, cacao, coffee, wheat and many other plants. It occurs in plants only in conjugated forms with chlorogenic acid.

The experimental mice were fed for one week and throughout the experimental period with powdered food mixed with caffeic acid (2%). The following treatments were included in the experimental protocol: 1) control (untreated), 2) DMSO (solvent control), 3) caffeic acid control, 4) caffeic acid + DMSO, 5) DMBA in DMSO, 6) caffeic acid + DMBA. Bone marrow samples were collected at 24, 48, 60, 72 and 96 hr after treatment with DMBA. The samples collected at 60 hr from DMBA treated mice showed the maximum number of micronucleated PCE (about

14/500 PCE). At the same sampling time the bone marrow samples from the mice fed with caffeic acid showed about 7 micronucleated PCE/500 PCE; i.e., the inhibitory effect of caffeic acid is about 50% at 60 hr (Fig. 2). A detailed report has been published elsewhere (Raj et al. (32)).

5. Ascorbic Acid

Ascorbic acid (Vitamin C) is widely distributed in the plant and animal kingdom. Good sources are citrus fruits, hip berries, acerola, fresh tea leaves, etc. Aside form its use as a vitamin, ascorbic acid or some derivatives are employed as antioxidants in foodstuffs, to prevent rancidity, to prevent the browning of cut apples and other fruit, and in meat curing.

Effect of ascorbic acid was tested against DMBA, BaP, CP and MMC induced chromosomal breaks in mice using bone marrow micronucleus assay. Ascorbic acid, dissolved in physiological saline, was injected to experimental mice one day before mutagen treatment. The dose of ascorbic acid was 520mg/Kg (80% LD_{50}). Next day the mice were injected with either DMBA (25mg/Kg), BaP (186mg/Kg), CP (43mg/Kg), or MMC (1mg/Kg). Bone marrow samples were collected at 24, 48 and 72. hr.

Our results indicated that ascorbic acid itself produced about 2.1 micronucleated PCE/500 PCE at 48 hr. Inhibitory effect of ascorbic acid was seen both in DMBA and BaP treated mice. The inhibition in DMBA treated mice was about 40% at 48 hr and 30% at 24 hr; whereas in BaP treated mice the inhibition was about 66% at 48 hr and 50% at 72 hr. The situation is reverse in CP and MMC treatments. Instead of inhibition, promotory effect was noticed especially at 48 hr when ascorbic acid was used along with the mutagens.

From these results one could say that ascorbic acid acts as an inhibitor against certain mutagens (DMBA and BaP) and as a promoter in combination with CP or direct acting mutagen MMC.

6. Disulfiram

Disulfiram (tetraethylthiuram disulfide) was first produced in 1881 and was used in the rubber industry to accelerate the vulcanization of rubber. Disulfiram was introduced in the 1930's into the medical world as a scabiescide. It is an off-white, odorless, crystalline powder and was found to be toxic to lower forms of life utilizing copper-containing respiratory enzymes. Intestinal worms were especially sensitive to disulfiram and hence, this was introduced as a vermicide. Disulfiram was proposed for the prophylaxis of chronic alcoholism.

Azomethane is a carcinogen and the oxymetabolite of azomethane produces neoplasia (Fiola et al., 33) and disulfiram offers raido-protection from the oxymetabolite neoplasia, indirectly inhibiting the metabolism of azomethane.

Lang et al. (34) demonstrated that disulfiram and its major metabolite, diethyldithiocarbamic acid, diminished the inductive effect of phenobarbital on cytochrome P-450 content and p-nitro-anisole-O-demethylation, while both compounds showed additive effect in inducing NADPH-dependent cytochrome reductase. These studies indicated that disulfiram acted as an inhibitor against certain promoters (e.g., phenobarbital). Therefore, we conducted experiments using disulfiram to see whether there would be any inhibitory action against DMBA, BaP, CP and MMC-induced chromosomal breaks, using the bone marrow micronucleus test.

Experimental mice were fed for 10 days on a diet containing 1% disulfiram and on the 11th day the mice were injected i.p. with mutagens. Bone marrow samples were collected at regular intervals. In the case of DMBA-induced chromosomal breaks, an inhibition of about 80% was noticed by disulfiram in the 48 hr sample. In the case of the other mutagens, no inhibitory activity was caused by disulfiram.

7. Metyrapone

Metyrapone (Metopirone, or 2-methyl-1,2-di-3-pyridyl-1-propanone) is used as a diagnostic tool for the determination of residual pituitary function in patients with hypopituritarism (Sprunt et al., 35; Meikel et al., 36). It has also been shown to inhibit a number of hepatic cytochrome P-450 dependent monooxygenations such as aromatic ring hydroxylation, and O- and N-dealkylation (Netter et al., 37; Kohl et al., 38; Hildebrandt, 39). This encouraged us to study the possible inhibitory effect of metyrapone against formation of chromosomal breaks caused by different mutagens.

Mice were injected with metyrapone (100mg/Kg) 48 and 24 hr before injecting either with DMBA (25mg/Kg) in DMSO, or BaP (150mg/Kg) in DMSO and the bone marrow samples were obtained at various intervals. Metyrapone, by itself, produced only about 1 MNPCE/500 PCE at all sampling points. DMBA treated mice showed, on average, a peak number of 7.0 MNPCE/500 PCE at 48 hr and metyrapone pretreated mice that received DMBA showed about an average of 7.4 MNPCE/500 PCE at the same sampling time. On the contrary, BaP-treated mice showed about 8 MNPCE/500 PCE at 48 hr and pretreatment with metyrapone followed by treatment with BaP showed about 3.6 MNCPCE/500 PCE which indicates about 56% inhibition against BaP.

8. Ethoxyquin

The effect of ethoxyquin, a non phenolic antioxidant, was studied against DMBA, BaP, CP or MMC treated mice for in vivo bone marrow chromosomal breaks. Experimental mice were fed with a powdered diet mixed with ethoxyquin (10g/Kg of food) for one week before injecting the mice with the above mutagens and continued till the end of experiment. Mutagens DMBA (25mg/Kg in DMSO), BaP (186mg/Kg in DMSO), CP (43mg/Kg in saline) and MMC (1mg/Kg in saline) were injected and the bone marrow samples were obtained at regular intervals starting

from 24 hr after mutagen treatment. Overall inhibition was observed at several time points in ethoxyquin fed mice treated with DMBA. However, 54% of inhibition was noticed particularly at the 24 hr sample. In the case of the CP treated mice, about 70% of inhibition was caused by ethoxyquin in the 24 hr sample (Fig. 3). In MMC treatment up to 40 hr sampling times (i.e., 24 and 40 hr) about 90% of inhibition was observed (Fig. 4). BaP treated mice did not react positively with ethoxyquin in showing inhibition. All the results pertaining to the inhibitors (2 to 8) are presented in Table 2 in consolidated form.

TABLE 2: Effects of Potential Chemical Inhibitors Against Mutagens in Bone Marrow Micronucleus Assay with Mice

Chemical Mutagen	Solvent for Mutagen	Inhibitor	Application	Extent of Inhibition % Bone Marrow Micronucleus
DMBA	Corn oil	5,6-benzoflavone	Corn oil, i.p.	50
BaP	DMSO	5,6-benzoflavone	DMSO, i.p.	66
BaP	Corn oil	5,6-benzoflavone	Corn oil, i.p.	100
DMBA	DMSO	Caffeic acid	2% in feed	50
DMBA	DMSO	7,8-benzoflavone	DMSO, i.p.	90
BaP	DMSO	7,8-benzoflavone	DMSO, i.p.	75
DMBA	DMSO	Ascorbic acid	Saline, i.p.	40
BaP	DMSO	Ascorbic acid	Saline, i.p.	66
DMBA	DMSO	Disulfiram	1% in feed,10 days	80
DMBA	DMSO	Metyrapone	i.p. pretreated	none
BAP	DMSO	Metyrapone	48 and 24 hr	56
DMBA	DMSO	Ethoxyquin	In feed, 1 week	54
CP	Saline	Ethoxyquin	In feed, 1 week	70
MMC	Saline	Ethoxyquin	In feed, 1 week	90

DMBA = 7,12-diemthylbenz(a)anthracene
BaP = Benzo(a)pyrene
CP = Cyclophosphamide
MMC = Mitomycin C

9. Corn Oil and Its Minor Constituents, and Sunflower Oil

The possible inhibitory effect of corn oil against polynuclear aromatic hydrocarbon-induced chromosomal breaks was suspected when studying the effect of beta-naphthoflavone (BNF) against DMBA. BNF dissolved in corn oil showed much more inhibiton than BNF in DMSO. In other treatments wherever corn oil was present, the inhibition was much more pronounced. Therefore, the effect of corn oil was studied sytematically, as follows.

8-10 weeks old female B6C3F1 hybrid mice were injected i.p. with corn oil (0.4ml/20g mouse) for two days consecutively, i.e., 48 and 24 hr before injecting them with promutagen DMBA. The following is a list of experimental treatments:

1) DMBA was dissolved in corn oil and injected in the mice which were pretreated with corn oil.
2) DMBA was dissolved in corn oil and injected in the mice which were pretreated with DMSO.
3) DMBA was dissolved in DMSO and injected in the mice which were pretreated with corn oil.
4) DMBA was dissolved with DMSO and injected in the mice which were pretreated with DMSO.
5) Mice were injected with DMBA dissolved in corn oil (without any pretreatment).
6) Mice were injected with DMBA dissolved in DMSO (without any pretreatment).

The bone marrow samples were collected at 24, 48, 72 and 96 hours after the last injection in the respective treatments. Reduction in number of MNPCE were observed at all points wherever corn oil was used as solvent. More than 50% inhibition was noticed at 72 hr sampling time. Pretreatment with corn oilshowed inhibition over untreated mice.

The minor constituents of corn oil were tested against DMBA-induced chromosomal breaks. Sunflower oil was included in our studies. Minor constituents of corn oil include gallates, α-tocopherol and β-sitosterol. Mode of treatment and the results are consolidated in Table 3.

TABLE 3: Corn Oil and Its Minor Constituents as Inhibitors Chromosomal Breakage Induced by DMBA In Vivo Bone Marrow Micronucleus Assay with Mice

Inhibitor	Pretreatment	Solvent for DMBA	MNPCE Sampling time, hr.	% Inhibition
Corn oil	-	DMSO versus Corn oil	72	>50
Corn oil	(0.4ml/20g mouse) i.p. for 2 days*	DMSO	72	35
Corn oil	i.p. for 2 days	Corn oil	48	70
Corn oil	i.p. for 2 days	Corn oil versus DMSO	72	60
Propyl gallate	(217mg/Kg)	DMSO	48 and 72	none
α- Tocopherol	1% in food for 4 days	DMSO	48	ca, 60
β- Sitosterol	(150mg/Kg) i.p. in 50% ethanol	DMSO	48	60
Sunflower oil	(0.4ml/20g mouse) i.p. for 2 days	DMSO	48	75

*Interperitoneal injections of inhibitor at 48 hr and 24 hr prior to DMBA treatment.

It is evident that mainly two natural ingredients, α-tocopherol and β-sitosterol, are the principal ingredients in corn oil that contributed to the inhibitory action against DMBA-induced chromosomal breaks. Not only corn oil, but also sunflower oil has the capacity to protect against clastogenic action caused by DMBA. Corn oil has 86% unsaturated fatty acids and 13% saturated fatty acids, whereas Unico Sunflower Oil has 65% unsaturated fatty acids and 12% saturated fatty acids and traces of beta carotene. Unsaturated fatty acids are recognized as essential dietary elements, and helpful to human health. Blood cholesterol levels are lower when unsaturated vegetable oils, instead of saturated fats, are used in the diet.

There are several reports dealing with the pros and cons regarding the effect of corn oil. Diets which provide a source of polunsaturated fatty acid enhance the in vivo metabolism of xenobiotics (Michalik et al., 40; Chadwick et al., 41) and result in alterations of pharmacologic responses to drugs (40; Wade et al., 42,43). The increased concentrations of cytochrome P-450 and other key components of the hepatic mixed function oxidase systems appear to be responsible for the enhanced in vitro and in vivo metabolism of drugs and other xenobiotics by animals fed these polyunsaturated fat diets (Chen et al., 44; Lam and Wade, 45,46; Lambert and Wills, 47). Corn oil and sunflower oil pretreament in our experiments must have caused induction of the mixed function oxidase system to the extent that it resulted in metabolism of DMBA as indicated in Table 3.

II. Abnormal Spermhead Assay

B6C3F1 male mice (Charles River Canada Inc.) were 11 to 14 weeks of age when used for sperm abnormality assay. The mice ranged from 24-27g in weight

and they were maintained under normal animal room conditions before and during experimental periods. All mice were fed with Purina Laboratory Rodent Chow and watered ad libitum. All the chemicals were injected i.p. and the sperm cell samples were collected from Cauda epididymides on the 35th day after the last injection and sperm smears were prepared according to Wyrobek and Bruce (20). At least 500 sperm cells were scored at a magnification of 400x and a number of abnormal sperm cells were counted, using the "normal and abnormal murine sperm chart" of Wyrobek et al. (19,21). Sperm abnormalities might permit a simple quantative assay for damage to the genetic material of the male germ line.

10. Ferulic Acid - Inhibiton of Mutagen-Induced Germ Cell Abnormality

Ferulic acid and caffeic acid have been shown to diminish the incidence of forestomach tumors of mice treated with BaP (Wattenberg et al., 48). Recently, Wood et al. (49) reported the inhibitory effect of naturally occurring plant phenols, including ferulic acid, against the mutgenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons using the Ames Salmonella assay and Chinese hamster cells in vitro. Newmark and Mergens (50) found that caffeic and ferulic acids were particularly effective in reactions with nitrite and thus blocking the formation of carcinogenic N-nitroso compounds. Since ferulic acid is widely distributed among fruits and vegetables that are regularly consumed by humans, a study was made of the possible inhibitory effect of ferulic acid against DMBA-, BaP-, CP- and MMC-induced abnormalities in germ cells.

The male mice received pretreatment by being fed on a powdered diet containing 2% ferulic acid for one week before receiving the appropriate mutagen. On the 35th day after the last injection, sperm samples were collected and the cells analyzed for abnormal sperm cells.

The following doses of mutagens were given to mice i.p.:

DMBA:	20mg/Kg	2 injections
BaP:	150mg/Kg	1 injection
CP:	45mg/Kg	1 injection
MMC:	0.8mg/Kg	4 injections.

The results are summarized in Table 4.

TABLE 4: Results from Action of Ferulic Acid on Mutagen-Induced Germ Cell Abnormality

Treatment	No. of mice treated	No. of mice surviving till 35th day	Mean no. of abnormal sperm/ 500 sperm±S.E.
Ferulic Acid (FA)	5	5	11.0 ± 3.4
DMBA	5	1	11.7
BaP	5	0	Toxic
CP	6	6	8.7 ± 2.6
MMC	5	5	7.5 ± 0.8
DMBA+FA	10	5	13.2 ± 1.8
BaP+FA	10	3	16.5 ± 3
CP+FA	10	8	9.9 ± 1.5
MMC+FA	10	7	17.2 ± 4.3

The results shown in Table 4 indicate that ferulic acid in the feed apparently produced a relatively high number of abnormal germ cells, comparable to that produced by DMBA alone and higher than that produced by CP and MMC, acting alone. BaP was toxic to mice at the dose employed. It is presumed that

if FA had no inhibiting effect on the above mutagens, the mean number of abnormal sperms would in a given combination be represented by the sum of FA plus mutagen. However, DMBA + FA yielded 13.2 instead of the expected total of 22.7; CP + FA, 9.9 instead of 19.7 and MMC + FA, 17.2 instead of 18.5. Therefore, it can be tentatively concluded that ferulic acid has an inhibitory effect on abnormal germ cells induced by DMBA and CP, but the results with BaP are obscured by its toxicity to mice at the dose used of 150mg/Kg.

11. α- and β-Naphthoflavones - Inhibitors of Mutagen-Induced Germ Cell Abnormality

Experimental mice were injected i.p. either with α-NF or β-NF (both 80mg/Kg) for two days consecutively at 24 hr intervals before injecting DMBA (20mg/Kg) for the next two days successively. Five mice were used in each treatment, except in the α-NF treatment where only 4 mice were used. Sperm cell samples were collected from cauda epididymides on the 35th day after the last injection and sperm smears were prepared.

The results are summarized in Table 5. The survival rate of treated mice was good except in two treatments. The survival was 3 out of 5 in DMBA alone, and 2 out of 5 in β-NF + DMBA. The range of abnormal sperm cells was more or less the same in untreated controls, solvent controls α-NF and β-NF. Number of abnormal sperm cells in DMBA treatment was high, ranging between 74-138 in different mice with an average of 108±18.6. Considerable reduction in number of abnormal cells was noticed both in α-NF (83%) and β-NF (60%) pretreated mice.

TABLE 5: Inhibitory Effect of α-Naphthoflavone (α-NF) and β-Naphthoflavone (β-NF) on DMBA-Induced Abnormal Sperm Cells.

Treatmen	No. Mice Used	No. Mice Survived*	Range of Abnormal Sperm Cells/500 Sperm Cells	Mean±S.E.
1. Untreated	5	5	6-14	9.2 ± 1.6
2. DMSO (solvent control)	5	5	7-20	13.6 ± 2.5
3. α-NF (2x)**	4	4	8-16	11.5 ± 1.7
4. β-NF (2x)	5	5	6- 12	8.8 ± 1.0
5. DMBA (2x)	5	3	74-138	108 ±18.6
6. α-NF(2x) + DMBA (2x)	5	5	7- 26	18.8 ± 3.4
7. β-NF(2x) + DMBA(2x)	5	2	40- 46	43.0 ± 3.0

* Mice survived til 35th day after last injection.
** 2x indicate 2 injections, 24 hr apart in two days.

Studies with flavones, such as α- and β-NF have shown that animals can be protected from some of the deleterious health hazards caused by PAHs. It was observed that α-NF acted as a potent inducer of increased mixed-function oxidase activity, resulting ultimately in inhibition of epidermal neoplasia caused by DMBA (Gelboin and Ts'O, 5; Slaga, 7; Wattenberg, 51). The clastogenic acitivty of DMBA was found to be inhibited by α-NF (Raj and Katz, 31) as well as by β-NF (Salamone et al., 28). Furthermore, pulmonary and skin tumors in mice and mammary tumors in rats, resulting from exposure to DMBA, were shown to be inhibited by prior treatment with benzoflavones (13).

PAIRWISE COMBINATIONS OF MUTAGENS IN BONE MARROW MICRONUCLEUS ASSAY

People are exposed to an environment which contains a mixture of pollutants, especially in the urban industrial air where contaminants include polynuclear aromatic hydrocarbons (PAH). The effect of paired mixtures of DMBA and BaP were studied, using the in vivo bone marrow micronucleus assay.

Preliminary experiments have been conducted with a combination of DMBA + BaP in treatment with doses of 15mg/Kg for DMBA and 95mg/Kg for BaP. The results are presesented in Table 6. These treatments at half the usual dosage produced less than the average maximum MNPCE of a single PAH exposure. Therefore, we repeated the experiments with normal doses, i.e., DMBA (30mg/Kg), BaP (186mg/Kg).

In 48 hr samples, the average number of MNPCE/500 PCE were 7.2±1.1 (DMBA), 7.4±1.6 (BaP) and 4.4±1.2 (DMBA + BaP), indicating an antognistic effect instead of an additive effect. These results confirm our earlier results.

TABLE 6: Pairwise Combination Experiments with Mutagens Bone Marrow Micronucleus Assays with Mice

Mutagen	Dose	Max. Average MNPCE/500 PCE	Sampling time, hr.	Remarks
DMBA	15m/gKg	5.8	48	
BaP	95mg/Kg	8.0	60	
DMBA + BaP		3.6	48	Non-additive
DMBA + BaP		6.2	60	(overlapping standard error bars)

Other sets of experiments were also conducted using paired mixtures. One set of mice was treated with a mixture of DMBA (30mg/Kg) and MMC (1mg/Kg), and another set was treated with BaP (186mg/Kg) and CP (45mg/Kg). In 48 hr samples, the average numbers of MNPCE/500 PCE were as follows: DMBA (7.2±1.1), MMC (7.2± 1.8), CP (8.0±2.0), DMBA + MMC (23.5±1.3), BaP + CP (21.0±1.0). The additive effect was 163% in the combinaiton of DMBA + MMC and 136% in the combination of BaP + CP. In the DMBA + MMC combination, out of 5 mice only 2 mice survived. Each mouse showed 21 to 26 MNPCE/500 PCE. The above results are summarized in Table. 7.

TABLE 7: Summary of Results from Combination Experiments Using In Vivo Bone Marrow Micronucleu Assay

Combination of Mutagens			Remarks
DMBA (30mg/Kg)	+	BaP (186mg/Kg)	Antagonistic or inhibitory effect
DMBA (30mg/Kg)	+	MMC (1mg/Kg)	163% additive effect at 48 hr
BaP (186mg/Kg)	+	CP (45mg/Kg)	136% additive effect at 48 hr

PAIRWISE COMBINATIONS WITH MUTAGENS IN ABNORMAL SPERMHEAD ASSAY

Experimental mice were injected i.p. with DMBA, BaP, CP or MMC individually and also pairwise DMBA + BaP, DMBA + CP, DMBA + MMC, BaP + CP, BaP + MMC and CP + MMC. The results are summarized in the following Table 8.

TABLE 8: Pairwise Combination Experiments with Mutagens Abnormal Spermhead Assay with Mice

Mutagen	Dose mg/Kg	Mean No. Abnormal Sperms/500 Sperms ±S.E.	Remarks
DMBA	25	1.35 ± 1.08	
BaP	200	6.5 ± 2.5	
CP	45	8.7 ± 2.6	
MMC	0.8	47.8 ± 5.6	
DMBA + BaP		Toxic	Mice died
DMBA + CP		27.2 ± 4.2	Additive
DMBA + MMC		40.3 ± 6.9	Non-additive
BaP + CP		21.5 ± 7.5	Additive
BaP + MMC		Toxic	Mice died
CP + MMC		45.0 ± 4.0	Non-additive

PROMOTERS

Arochlor and Phenobarbital - Bone Marrow Micronucleus Assay

Arochlor and phenobarbital were tested against DMBA-treated mice to see whether they would act as promoters. In this case, the promutgen DMBA (25mg/Kg) was injected 24 hr before injecting the mice with either Arochlor or

phenobarbital. The doses of Arochlor and phenobartibal were 0.84ml/Kg (50% of LD_{50}) and 85mg/Kg (50% of LD_{50}) respectively.

The results indicated that in Arochlor treatment the highest mean number of micronucleated PCE/500 PCE was less than 1 at 48 hr. At the same time point the sample with DMBA alone showed an average number of 5.5 micronucleated PCE/500 PCE. Arochlor plus DMBA treatment showed about 40% excess of micronucleated PCE/500 PCE over DMBA treatment alone. This result indicates that Arochlor acts as a mild promoter in the presence of DMBA. Studies with Arochlor, tested against BaP-treated mice, did not cause any promotory effect.

Phenobarbital did not show any promotory effect with DMBA. However, many mice died in the experiment when phenobarbital was used. So we had to restrict ourselves to the 48 hr samples. In general, we observed that most of the bone marrow samples had more white blood cells compared to PCE wherever either Arochlor or phenobarbital was involved.

Hexachlorobenzene - Bone Marrow Micronucleus Assay

Hexachlorobenzene (HCB, perchlorobenzene) is a chlorinated benzene of increasing toxicological and environmental concern (Morita, 52; Mumma and Lawless, 53; Quinliven et al., 54; Winteringham, 55). HCB is a hazardous component of certain industrial chemical wastes. HCB is used as a fungicide primarily to control bunt of wheat. Mismanagement of HCB wastes and use of products have resulted in several serious incidents of environmental contamination (U.S. EPA, 56).

HCB has been found in water, soil and selected aquatic organisms along the lower Mississippi River in Louisiana. Highest levels were found downstream from

heavily industrialized areas (Laska et al., 57). Air immediately adjacent to production facilities has been shown to have concentrations from 1.0 to 23.6 $\mu g/m^3$.

HCB has also been identified in surface water and industrial effluents in U.S.S.R., Germany and Italy (Leoni and D'Arca, 58). HCB has been found in fish from a number of river systems in Canada (Zitko, 59). The carcinogenic activity of HCB in Syrian golden hamsters was demonstrated by Cabral et al., 60).

Mice wre injected either with DMBA (25mg/Kg) or BaP (186mg/Kg). After 24 hr these mice were injected with HCB(80mg/Kg) in DMSO and the bone marrow samples were collected at 24, 48 and 72 hr. At 24 hr, the average number of MNPCE/500 PCE was 5.8 in DMBA, 2.0 in HCB, 2.8 in BaP, and 11.0 in DMBA + HCB and 6.8 in BaP + HCB. These results indicated a promotory action of HCB against DMBA as well as BaP. However, an analysis of the results from the 48 hr samples indicated no promotory but instead an inhibitory effect. Presumably, the promoting action of HCB is evident for only 24 hr after injections and decreases after 48 hr or later (Figs. 5,6).

Hexachlorocyclohexane

Mice were treated either with DMBA (30mg/Kg) or BaP (150mg/Kg). Hexachlorocyclohexane (4mg/Kg) was injected, i.p., to the above mice after 30 minutes. Bone marrow samples were collected at 24, 48 and 72 hr. The average number of MNPCE/500 PCE were 8.4±0.9 in the 48 hr sample from the mice that received DMBA and 14.6±3.4 in the mice that received hexachlorocyclohexane. About 70% promotory effect was seen in the 48 hr sample from the mice that received both DMBA and hexachlorocyclohexane.

In the case of mice that received BaP and hexachlorocyclohexane, an 89% promotory effect was observed in the 72 hr sample, compared to the mice that received only BaP.

The above mentioned results are summarized in the following table.

TABLE 9: Potential Promoters of Mutagens
Bone Marrow Micronucleus Assay

Promoter	DMBA	BaP
Arochlor (PCB)	40% excess MNPCE	No effect
Phenobarbital	No effect	-
Hexachlorobenzene	Promotion at 24 hr Inhibiton at 48 hr	Promotion at 24 hr Inhibition at 48 hr
Hexachlorocyclohexane	70% Promotion at 48 hr	89% Promotion at 72 hr

Salmonella Typhirmurium Assay

This assay has been used to test the following potential inhibitors against BaP and MMC:- butylated hydroxyanisole, ethoxyquin, propyl gallate, ascorbic acid, caffeic acid, ellagic acid, ferulic acid, metyrapone, alpha-naphthoflavone and disulfiram. These inhibitors were tested in the presence and absence of S-9, using bacterial strain TA-98. (The procedure recommended by Ames et al. (16,17) has been followed.) However, a new Salmonella strain, TA-102, which is responsive to MMC, has been used wherever MMC is involved. Furthermore, we tested the effect of BaP, DMBA and CP using TA-102 strain in the presence and absence of S-9.

TA-102 is a new strain which was constructed primarily for detecting mutagens that require an intact excision repair system. TA-102 contains the R-factor plasmid, pKM101. It also contains the multicopy plasmid, pAQ1, which carries the G428 mutation and tetracycline resistance gene. TA-102 contains the ochre mutation $\frac{-TAA-}{-ATT-}$ in the his G gene. This strain detects efficiently a variety of mutagens such as formaldehyde, glyoxal, various hydroperoxides, bleomycin, phenylhydrazine, X-rays, UV light, streptonigrin and cross-linking agents, such as psorolens and mitomycin C (Maron and Ames, 61). (All the bacterial test strains have been kindly supplied by Dr. B.N. Ames.)

Effect of Butylated Hydroxyanisole (BHA) Against BaP

5µg of BaP have been used per plate in the controls as well as against BHA. Average number of revertants per plate were 193±7. BHA at the highest dose of 250µg showed toxicity. BHA showed an inhibitory effect at the highest non-toxic dose of 125µg/plate. Since the 250µg dose showed toxicity, 125µg may be, in fact, toxic and was not detected. Therefore, the amount of BHA was reduced very much in further experiments and the concentrations ranged between 15µg - 120µg/plate. Average number of revertants were 109±8 with 15µg, 65±3 with 30µg, 123±12 with 60µg and 112±5 with 120µg/plate of BHA. In this experiment, BaP controls gave an average number of revertants of 88±9. If we assume that the inhibitory effect has been seen with 30µg dose of BHA, the inhibitory effect should be almost doubled at 60µg dose. This was not the case. Hence it has been decided that BHA is not an inhibitor against BaP in the Salmonella assay.

Effect of Ethoxyquin Against BaP

BaP controls in this experiment gave 134±2 revertants on average. 125µg, 250µg, 500µg and 1000µg of ethoxyquin doses were tested against BaP using TA-98 strain. The average number of revertants per plate were 134±17 with 125µg, 106±3 with 250µg, and 90±11 with 500µg. No revertants were noted with 1000µg. The last two doses were found to be toxic. On the whole, no inhibitory effect of ethoxyquin was noticed.

Effect of Propyl Gallate Against BaP

In this experiment, BaP controls gave 145±11 revertants on average. 125µg, 250µg, 500µg and 1000µg doses of propyl gallate were tested against BaP. Highest dose of 1000µg/plate was found to be toxic. The average number of revertants per plate were 127±12 with 125µg, 148±9 with 250µg and 121±9 with 500µg/plate. These results indicate that propyl gallate is not an inhibitor against BaP in this assay.

Effect of Ascorbic Acid Against BaP

BaP controls gave 133±7 revertants on the average. The doses of ascorbic acid were 15, 30, 60 and 120µg/plate. The average number of revertants were 84±5 with 15µg, 89±1 with 30µg, 126±8 with 60µg and 117±3 with 120µg/plate. Ascorbic acid, by itself, gave 50±10 revertants with 120µg dose. On the whole, some inhibitory effect has been noticed.

Results with caffeic acid, ellagic acid, metyrapone, ferulic acid, alpha-naphthoflavone and disulfiram showed varying inhibitory effects in the Ames Salmonella assay against TA-98 bacterial strain. The results are presented in Table 10.

TABLE 10: Effect of Chemical Inhibitors Against BaP Salmonella Typhimurium Assay with TA-98 and S-9

Chemical	Maximum Dose	Revertants/Plate $\bar{X}$ ± S.E.	% Inhibition
BaP	5 µg	135±4	
Caffeic acid	4000nmol	83±5	40
Ellagic acid	4000nmol	80±13	41
Metyrapone	40µg	34±7	75
Controls			
DMSO	0.1ml	43±3	
Caffeic acid	4000nmol	27±2	
Ellagic acid	4000nmol	18±3	
Metyrapone	40µg	31±3	
BaP	5µg	185±9	
Ferulic acid	4000nmol	127±6	31
α-Naphthoflavone	40µg	93±9	50
Disulfiram	40µg	85±8	54
Controls			
Ferulic acid	4000nmol	53±2	
α-Naphthoflavone	40µg	52±6	
Disulfiram	40µg	53±3	
DMSO	0.2ml	61±6	

The following conclusions could be drawn from the above results.

1) Average number of revertants/plate with BaP alone varied from 135 to 185 in two different experimental runs. Reversion rate to TA-98 is about 40, so BaP has shown mutagenicity, with an increase in the reverion rate of 3 to 4 times.

2) An inhibitory effect was shown by all potential inhibitors.

3) The dose-response of the chemicals was not linear.

The above results with the Salmonella assay and the *in vivo* bone marrow micronucleus and abnormal sperm assays have provided clues to the possible inhibitory action of chemicals that are antioxidants, phenols and sulfur-containing compounds.

BaP, CP, DMBA and MMC were tested with and without the presence of S-9 using TA-102 bacterial strain. BaP gave negative resonse without S-9 and positive repsonse with S-9. CP and DMBA showed negative response with and without S-9. MMC showed a linear response on TA-102 with and without S-9. However, the response without S-9 was higher than that with S-9.

Caffeic acid, metyrapone, α-naphthoflavone, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ellagic acid, ascorbic acid, propyl gallate, ferulic acid and ethoxyquin were tsted against MMC using TA-102. None of the above mentioned putative inhibitors showed any inhibitory action against MMC.

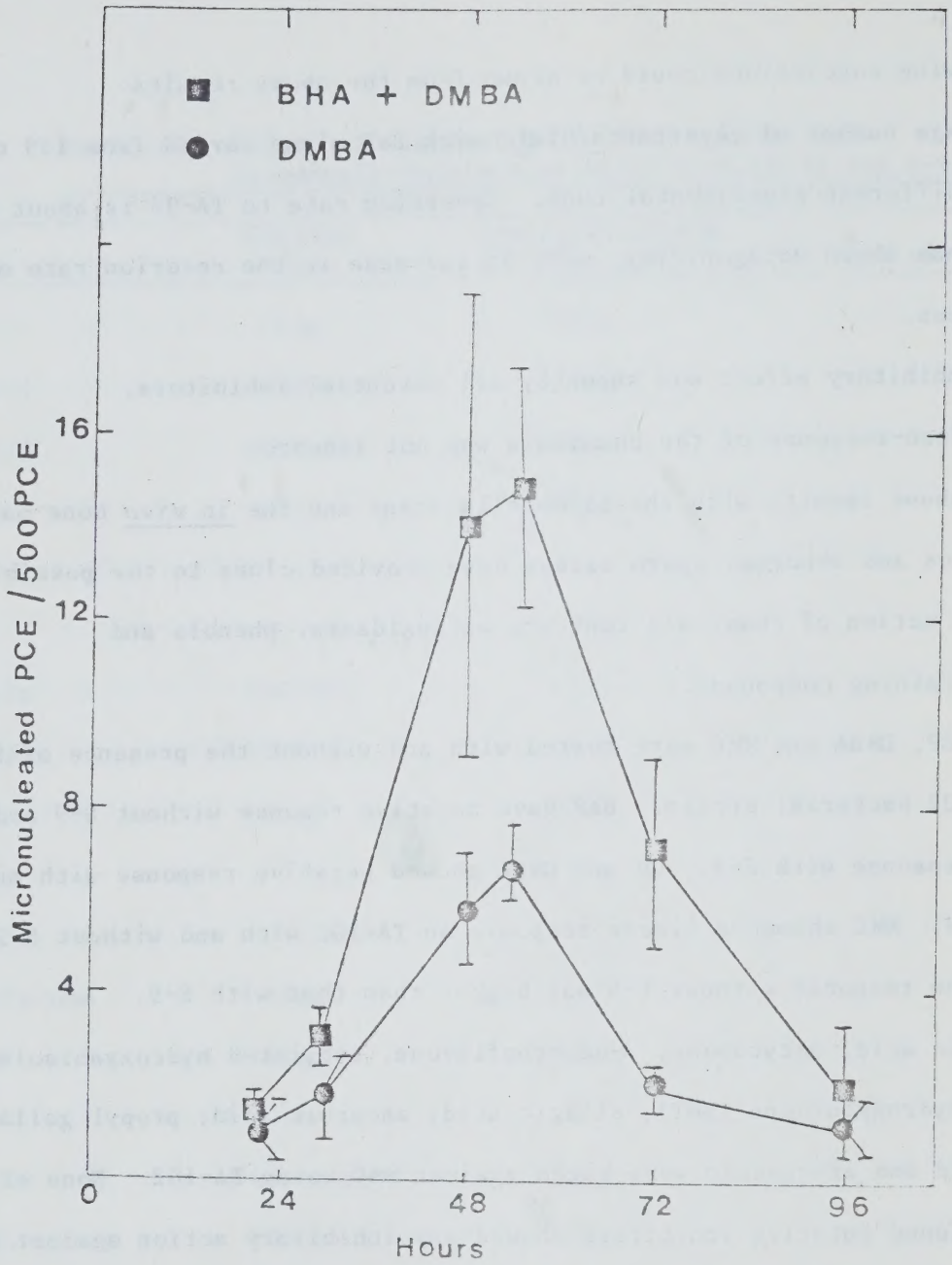

Fig 1. Results of bone marrow micronucleus assay of mice fed a powdered food mixed with butylated hydroxyanisole (BHA) and treated i.p. with DMBA in DMSO. BHA in the presence of DMBA acted as a promoter.

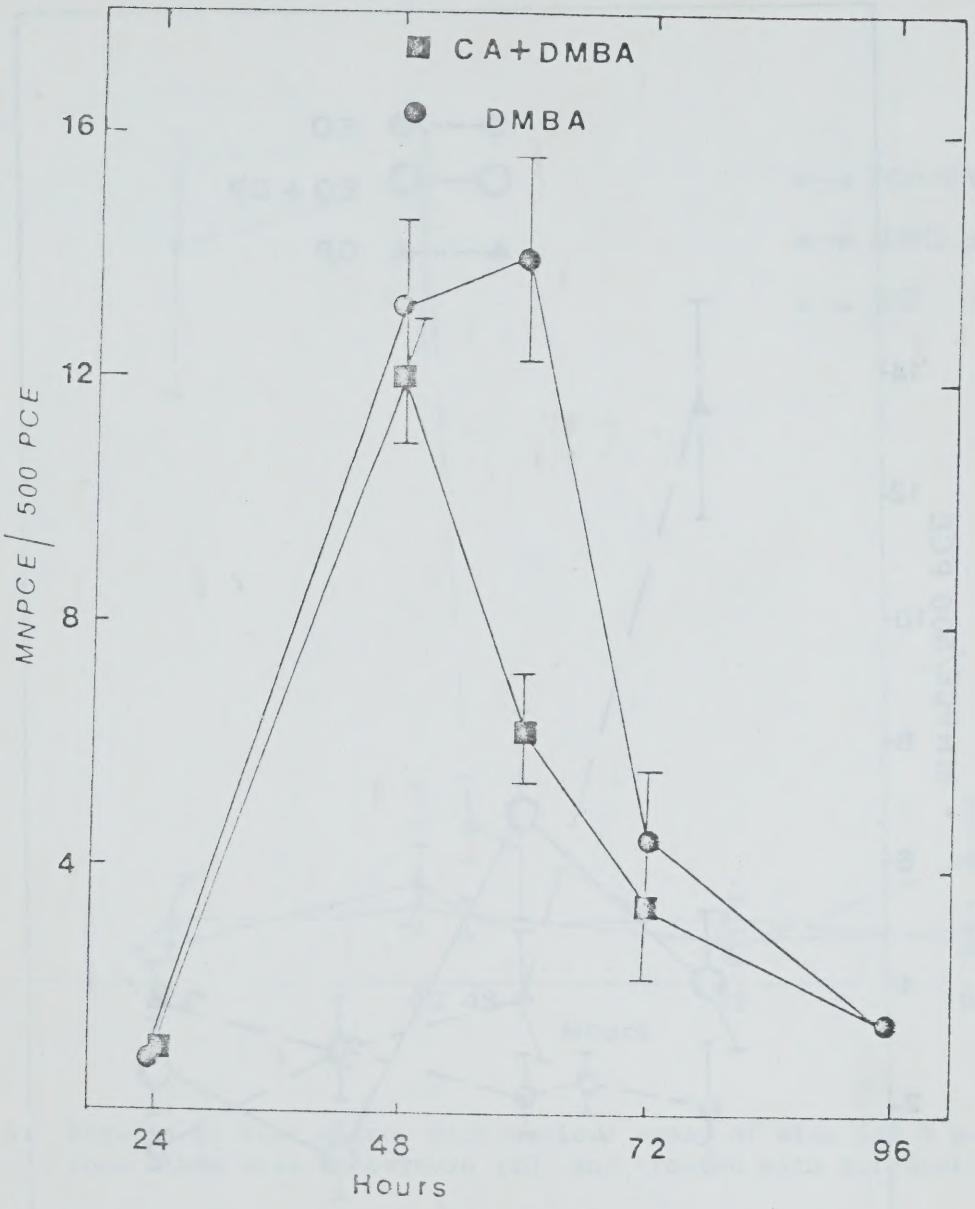

Fig. 2. Results of bone marrow micronucleus assay of mice fed a powdered food mixed with 2% caffeic acid and treated i.p. with DMBA in DMSO. Caffeic acid (CA) inhibits action of DMBA by about 50% at 60 hr sampling time.

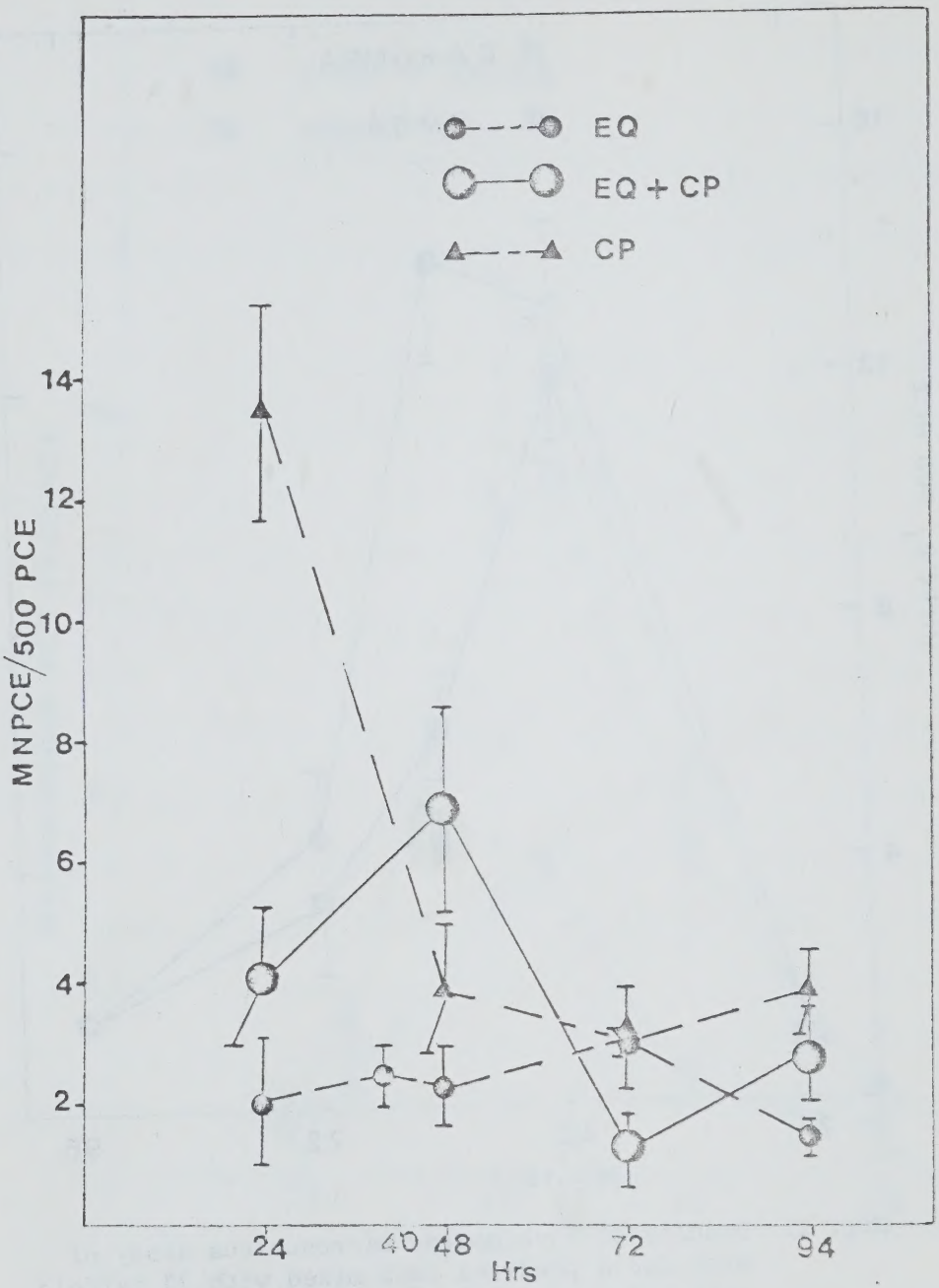

Fig. 3. Results of bone marrow micronucleus assay of mice fed a powdered food mixed with ethoxyquin (EQ) and treated with cyclophosphamide (CP).

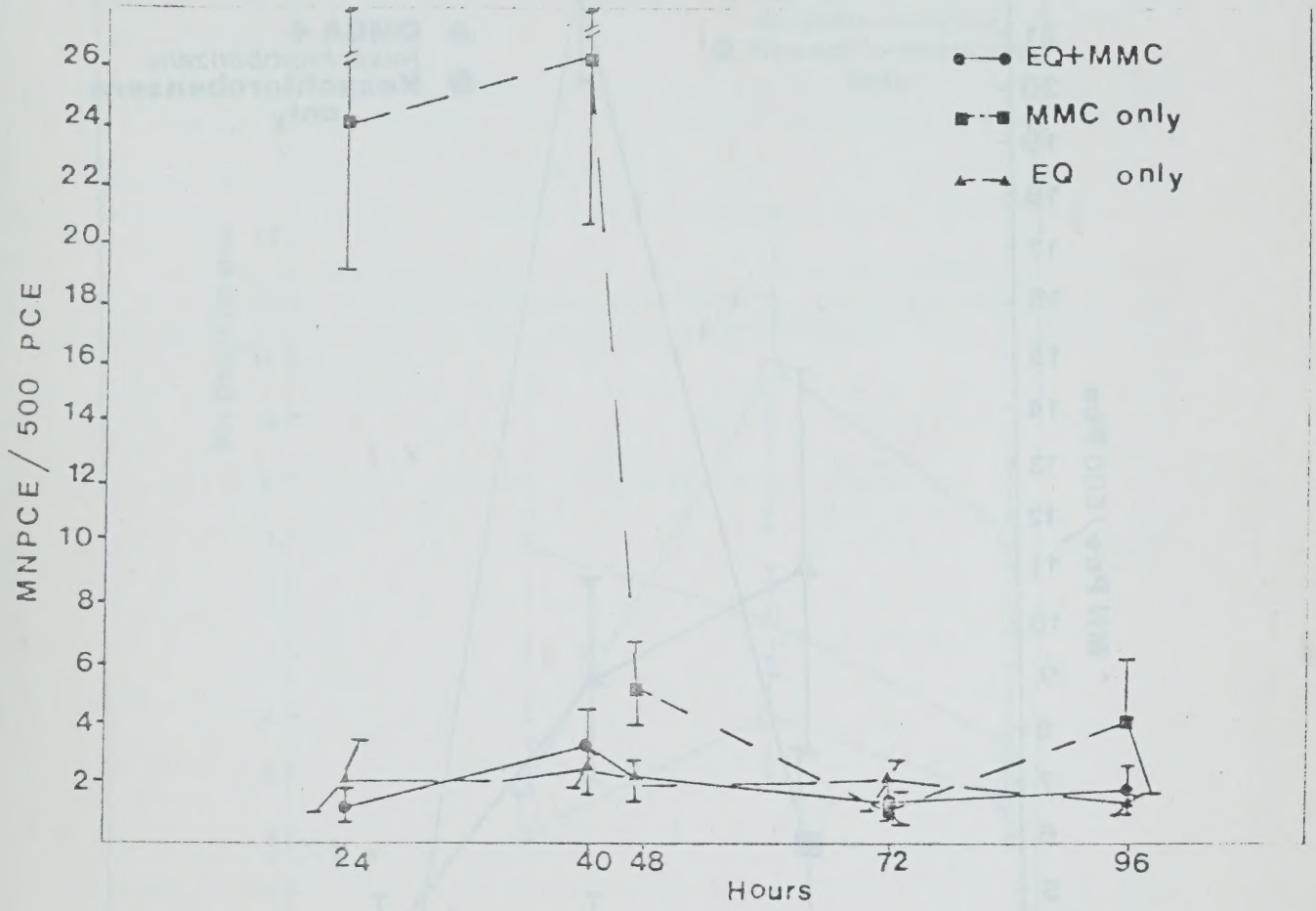

Fig. 4. Results of bone marrow micronucleus assay of mice fed a powdered food mixed with ethoxyquin (EQ) and treated with mitomycin C (MMC).

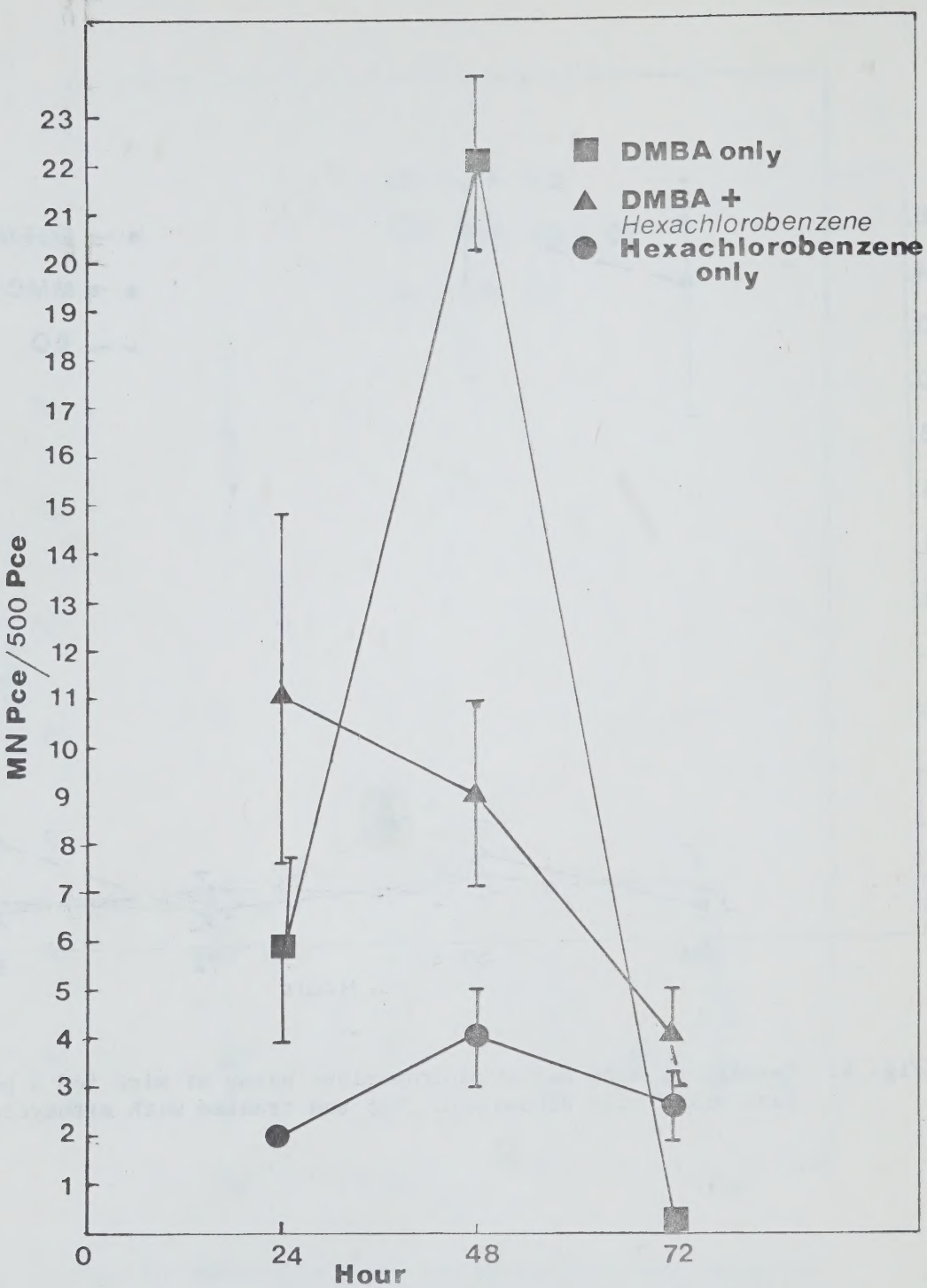

Fig. 5. Results from bone marrow micronucleus assay in mice treated with DMBA and hexachlorobenzene.

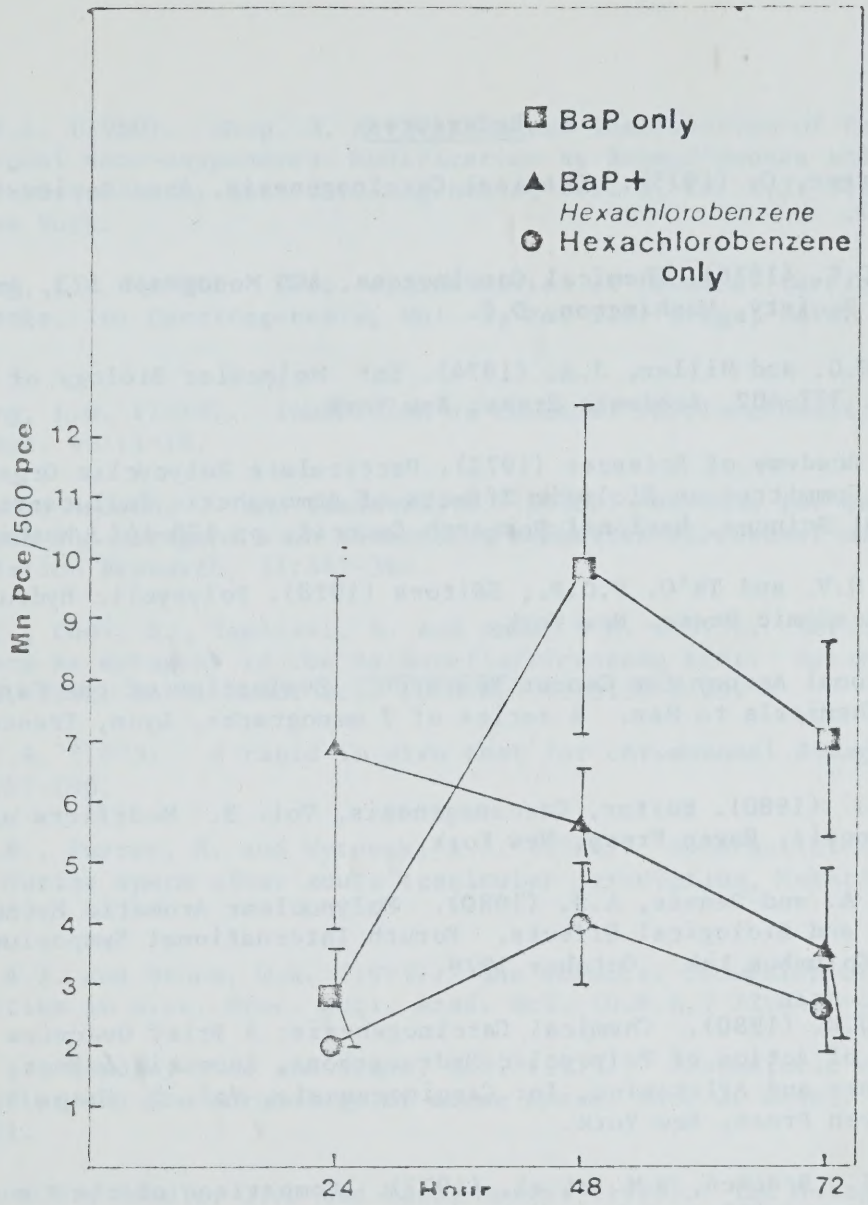

Fig. 6. Results from bone marrow micronucleus assay in mice treated with BaP and hexachlorobenzene.

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Collaborative Study on Short-Term Tests for Genotoxicity and Carcinogenicity

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Introduction

In 1976 the Health and Safety Executive (HSE) and the Medical Research Council (MRC) of the United Kingdom commissioned a programme to develop short-term mutagenicity tests to screen for possible carcinogens. Shortly thereafter, scientists from Imperical Chemical Industries (ICI) joined the programme and these groups agreed upon a combined approach. They believed that the best method of comparing the utility of different assays would be to test pairs of structurally related compounds, one carcinogenic, the other not, in the assay systems being assessed.

ICI agreed to supply the purified compounds and they selected 25 carcinogens and 17 non-carinogens, which included 14 paired compounds, for the study.

Since the available quantity of each chemical exceeded the requirements of the programme, the National Institute of Environmental Health Science (NIEHS) in the U.S.A. was invited to join the study with the intention of providing a wider variety of mutagenicity test systems. With the subsequent inclusion of several Japanese laboratories under the auspices of Japan's National Cancer Center Research Institute, the first international study on mutagenesis/carcinogenesis was initiated.

In the ensuring years, 42 test compounds were tested on 35 assay systems in some 60 laboratories. The study produced several results, including the identification of several previously unsuspected carcinogens, and tested the efficacy of different batteries of short-term mutagenicity tests. Unfortunately, the range of results with certain chemicals and assays systems precluded the identification, at that time, of a single

battery of short-term tests which would provide unequivocal identification of carcinogens. The other successes of the study, however, led to the establishment of a second international study.

This latter study, in which we are participants, is designed to correct the shortcomings of the initial study and expand its scope. The principal scientists from ICI and NIEHS who were originally involved in the organization and direction of the initial study are associated in a similar capacity with the present programme. This study, however, involved many additional nations and laboratories and the study is now jointly sponsored by the World Health Organization (WHO), the United Nations Environment programme and the International Labour Office.

Our participation involves testing four assay systems: the *in vivo* mammalian bone marrow micronucleus assay, the *in vivo* mammalian abnormal spermhead assay, the *in vitro* sister chromatid exchange assay and the replicative/killing assay. Four to six chemicals were to be tested on the two *in vivo* assays and ten chemicals on the *in vitro* assays. In addition, six chemicals, which were not part of the international study, were supplied by the Ontario Ministry of the Environment for testing on the *in vivo* assays.

Materials and Methods

Chemicals

(a) WHO Chemicals

All those chemicals which formed part of the international study were synthesized and provided in pure form by ICI. In each case, all testing labs received samples from a single synthesis to ensure comparability.

(b) MOE Chemicals

All chemicals provided by the MOE were obtained from commercial suppliers in the purest form commercially available.

All chemicals were dissolved or diluted in dimethyl sulfoxide. All other chemicals were obtained from regular suppliers in the highest purity available.

Animals

The B6C3F1 hybrid mouse was used in both in vivo assays. All mice were purchased from Canadian Breeding Farm and Laboratories Limited, Quebec. Mice used in a particular experiment were always selected from a single shipment and were age matched in all cases.

Chinese Hamster Ovary (CHO) cells were kindly supplied by Dr. Richard Marshall of York University.

Assays

In vivo

Bone Marrow Micronucleus Assay

Micronuclei are small pieces of nuclear material found in the cytoplasm of dividing cells. The material arises primarily from chromosome fragments that are not incorporated into daughter nuclei at the time of cellular division. Clastogens are mutagenic agents which are capable of breaking chromosomes and producing micronuclei, and thus are detectable by a micronucleus assay. The micronucleus assay used in this study was a slight modification of the mammalian bone marrow micronucleus assay described by Salamone et al (1980) and Salamone and Heddle (1983) in which polychromatic erythrocytes are used as the target cells. Mice

were treated once or on two consecutive days with a given dose of the test chemical. Approximately 24, 48 and 72 hr after the final treatment, samples were taken to determine the frequency of micronucleated bone marrow polychromatic erythrocytes.

For the WHO chemicals, a series of doses was tested with each chemical (rather than a single dose) to provide dose-effect data.

An equal number of male and female mice of the same age were used for each treatment. Samples were taken by first sacrificing the mice by cervical dislocation, removing a femur and stripping it of muscle (Salamone et al, 1980). The bone marrow was then removed by making a small opening at the iliac end of the femur and introducing the pointed shaft of a 2.5 cm pin into the femur at the epiphysial end. As the pin was slowly pushed and twisted into the marrow canal, the marrow exuded out the hole at the iliac end. The marrow was placed on a clean microscope slide and a drop of fetal bovine serum was then added. Using the edge of a clean slide, the marrow was mixed with the serum until homogenous and then spread as a smear. These bone marrow slides were then fixed in absolute methanol and stained with a 5% solution of Giemsa (BDH) (Salamone et al, 1980). The number of micronucleated polychromatic erythrocytes (PCE) per 500 PCE was then determined for each slide and results from mice treated with the test chemical were compared with those from non-treated and solvent treated mice.

Spermhead Abnormality Assay

The assay used in this study was that recommended by Wyrobek et al (1983). Male mice 11 weeks or older were given daily interperitoneal injection of the test chemical for five days. The protocol included five or six treatment groups plus zero, and solvent control groups. The effectiveness of the assay was routinely monitored by the injection of parallel experiments of known mutagens. Thirty-five days after the final treatment, the mice were sacrificed by cervical dislocation and both epididymides removed and placed in phosphate buffer. The tissue was minced and then stained for 20 min in a 1% Eosin solution. Stained sperm were spread on microscope slides, allowed to air dry and mounted with DPX (BDH Chemical). The number of abnormal sperm per 500 sperm was then measured.

In Vitro

Sister Chromatid Exchange (SCE)

The SCE assay monitors the frequency of exchange between chromatid arms of the same chromosome. Each sister chromatid can be selectively stained so that one appears dark while the other appears light. Exchanges can then be easily recognized. For this study, Chinese Hamster Ovary (CHO) cells were used and the basic method followed was that described by Latt et al (1981). CHO cells were routinely cultured in Eagles MEM buffered medium (DIFCO) supplemented with 10% fetal calf serum.

For testing purposes, culture flasks were initially seeded with 1.5×10^5 cells and allowed to grow to approximately 75% confluence. The cells were then freed and plated on 60 mm plates at 4.8×10^5 cells per plate and allowed to grow overnight. The old medium was then removed and fresh serum-free medium containing the test chemical or the test chemical plus 59 (metabolic activation mixture) was added. The cultures were incubated 1 h at 37°C following which the medium was removed, the cells washed with Hank's BSS (Gibco) and then recovered with fresh medium containing 20 μM BUDR (bromodeoxyuridine).

Twenty-four hours after the addition of BUDR, 0.1 ml of colchicine (10^{-4} M) was added. Two hours later, the BUDR colchicine medium was removed and the cells were harvested. Cells were trypsinized and 74 mM KCL was added for 6 min. The cells were then washed 3 times in Carnoys' (glacial acetic-alcohol) fixative, stained with Hoechst for 12 min, illuminated 22 h and then stained 5 min with 20% buffered Giemsa. The average frequency of SCE per 25 complete sets of metaphase chromosomes were scored. Results from cells treated with the test chemical were compared with those from non-treated or solvent treated cells.

Replicative/Killing Mutagenicity Assay

The Replicative/Killing assay measured the mutagenicity of chemical compounds using a genetically engineered strain of the bacterium Escherichia coli. This particular bacterial strain contains an incomplete virus particle which, in the inactive state at 30°C, resides symbiotically in the bacterial cell. Activation of the virus can be achieved by an increase

in the temperature of the culture to 42°C and this activation ultimately kills the bacterial cell. Mutagenic agents, on the other hand, may cause damage to the viral genetic material and prevent either its activation or expression. Bacterial cells of this strain, in which the virus has been mutated, will not be killed by a temperature shift and will develop into colonies.

In the Replicative Killing assay, a culture of bacterial cells is mixed with the test chemical, both in the presence and absence of metabolic activation enzymes (S9). A range of concentrations of the chemical is tested. Following a suitable incubation period, aliquots of the bacterial suspension are plated on a nutrient agar medium which is then incubated for 48 hr at 42°C. Bacterial colonies developing on the plates are counted and compared to counts on control plates containing a similar bacterial suspension which was not exposed to the test substances. A chemical possessing mutagenic properties should induce a dose-related increase in surviving colonies which exceeds, by a minimum of two fold, those on control plates.

LD_{50} and Toxicity

To minimize the frequency of false negative results, it is desirable to test chemicals at the highest tolerable dose. This dose is generally determined from the toxicity of the chemical, toxicity being expressed as some lethal dose percentage (e.g., LD_{10}, LD_{30}, LD_{50}), i.e., that dose which is expected to cause the death of a given percentage of the

population of test organisms. In this report, the toxicity in the *in vivo* assays is expressed as the LD_{50}, while for the *in vitro* studies, a visual measure of cell growth inhibition was used to ascertain the maximum test dose, i.e., the lowest dose at which cell growth inhibition was clearly discernible.

LD_{50}'s were calculated from the tables and formulae of Weil (1952). Each compound was tested at four or five doses, with the doses being separated by a constant factor (usually between 1.2 and 2). A uniform number of mice (usually 3 to 6) were injected at each of the selected doses and the number of deaths at each dose was determined daily over a 7 day period. For the micronuclei assay, the maximum dose tested was generally 80% of the LD_{50}, while for the abnormal spermhead assay 50% of the LD_{50} was routinely the maximum dose.

For the sister chromatid exchange assay, the toxicity of each chemical was checked using micro- and millimolar concentrations. A uniform number of CHO cells was allowed to grow in each well of a deep well plate (12 wells/plate). Various concentrations of the test substance were then added to each well. After 24 h each well was visually checked to determine the relative density of the cell growth. The lowest dose which exhibited some degree of toxicity was selected as the maximum test dose.

Toxicity with the RK assay is inherent within the assay as explained above.

Results and Discussion

The approximate LD_{50}'s of the chemicals listed in the *in vivo* assays are presented in Table 1. In interpreting this data, however, several points must be considered--most importantly, solvent effects. Many organic solvents are toxic in animal systems and thus, unless a chemical can be easily dissolved in water or physiological saline, dimethylsulfoxide (DMSO) becomes the solvent of choice. DMSO, which is a good universal solvent, is only mildly toxic to animals; however, it is not necessarily the best solvent for certain substances. For example, in the cases of atrazine and mirex, it was difficult to administer enough chemical to the animal in diluted form as DMSO was not a good solvent. In these cases, slurries of the test chemical and DMSO were injected. This required larger bore needles than usual and may have resulted in significant loss of chemical either from loss at the wound site or from retention in the syringe. Thus, for these two chemicals in particular, only estimated limits for the LD_{50} are reported.

It is of interest to note that the analogs, 2-acetylaminofluorene (2AAF) and 4-acetylaminofluorene (4AAF), have strikingly different LD_{50}. 2AAF has been reported to be the mutagenically more active of the two compounds, both *in vitro* and *in vivo*, and yet it was many times less toxic.

A summary of the *in vivo* mutagenic results for both the bone marrow micronucleus and the abnormal spermhead assays is presented in Table 2. In those cases where the data are equivocal, confirmation assays are underway.

TABLE 1: LD_{50} TEST RESULTS ON CHEMICALS TESTED IN VIVO

Compound	Approximate LD_{50}
WHO Compounds	
4-Acetylaminoflourene	364 mg/Kg
2-Acetylaminofluorene	2,220 mg/Kg
Benzo(a)pyrene	232 mg/Kg
Pyrene	514 mg/Kg
Hexamethylphosphoramide	
O-Toluidine	
MOE Compounds	
Atrazine	300-700 mg/Kg [a]
Chlorobenzene	65 mg/Kg
Ethylene dichloride	∿250 mg/Kg
Mirex	150-400 mg/Kg [a]
Pentachlorophenol	46 mg/Kg
Trichloroethylene plus stabilizer	125 mg/Kg
Trichloroethylene minus stabilizer	60-100 mg/Kg [b]

[a] Due to solvent difficulties, it has not yet been possible to determine a narrower toxicity range.

[b] Toxicity testing incomplete.

TABLE 2: SUMMARY OF IN VIVO MUTAGENICITY TEST RESULTS

Compound	Assay	
	Micronucleus	Abnormal Spermhead
WHO Compounds:		
4-Acetylaminofluorene	TBS	-
2-Acetylaminofluorene	TBS	+[a]
Benzo(a)pyrene	+	TBS
Pyrene	-	?+
Hexamethylphosphoramide	+	NT
O-Toluidine	?+	NT
MOE Compounds:		
Atrazine	INC	INC
Chlorobenzene	-[a]	-
Ethylene Dichloride	+[a]	-
Mirex	-[a]	TBS
Pentachlorophenol	+[a]	-
Trichloroethylene plus stabilizer	-	-
Trichloroethylene minus stabilizer	INC	INC

[a] Unconfirmed

NT No test, test not conducted as these chemicals were dropped from WHO programme.

TBS To be scored, experiment finished but slides not scored.

INC Incomplete, a full test has not been conducted

\+ Positive

\- Negative

?+ A weak positive response, requires confirmation at higher doses.

For the WHO compounds, micronucleus assay results are available for four of the compounds and three results are available based on the sperm assay (one compound was tested in both). Benzo(a)pyrene (BaP), hexamethylphosphoramide (HMPA) and O-toluidine gave positive results in the micronucleus assay while pyrene gave a negative result, i.e., pyrene is non-clastogenic. The data obtained with both latter compounds, i.e., O-toluidine and pyrene, are, however, not completely conclusive and require confirmation.

In the other in vivo assay system, i.e., abnormal spermhead assay, 2AAF gave positive results (i.e., acted mutagenically) while 4AAF did not. Pyrene, at high doses, also gave positive results (note: contrast with micronucleus assay) but again these results are equivocal and subject to confirmation.

In in vitro assay systems, both benzo(a)pyrene and pyrene have been reported to give positive responses, i.e., mutagenic with BaP producing the stronger response (de Serres and Ashby, 1981). In vivo assays have identified BaP as a fairly strong mutagen but pyrene has not been detected as a mutagen until now. The results presented here suggest that pyrene may indeed induce in vivo mutagenesis in gonadal tissue. These results need verification, but it is of interest to note that Topham (1981) reported an almost identical response with pyrene on the sperm assay.

2AAF and 4AAF have produced mutagenic patterns very similar to that of BaP and pyrene, respectively. The abnormal spermhead results for these compounds are, therefore, not totally unexpected.

HMPA has not produced a positive mutagenic response in *in vitro* systems but has produced strong mutagenic results *in vivo* (de Serres and Ashby, 1981). The micronucleus data presented here verify HMPA's mutagenic response *in vivo*. In contrast, O-toluidine has, in the past, been reported to produce positive results in *in vitro* assays, but negative results in *in vivo* assays (i.e., a response pattern equivalent to that shown by pyrene and 4AAF). In our hands, however, O-toluidine produces a weak positive response in an *in vivo* assay. Confirmation of this result will reflect well on the current protocol for the micronucleus assay. Unfortunately, during the course of our work, both O-toluidine and HMPA were dropped from the WHO programme and work on these compounds will accordingly be delayed.

Of the seven MOE compounds tested, only 1,2-dichloroethane and pentachlorophenol have so far shown positive clastogenic responses, and none of the compounds appear to be mutagenic to gonadal tissue. Most of the micronucleus assay data the subject to confirmation, however. Furthermore, initial tests on atrazine and trichloroethylene minus stabilizer have not been completed, therefore, it is possible that one or two other of these chemicals may prove mutagenic in *in vivo* systems.

The *in vitro* results are presented in Table 3. Since the metabolic enzymes necessary for the conversion of promutagens into mutagens are not inherently present in CHO cells or *E. coli*, they must be added to the reaction mixture. Each chemical was tested with (+S9) or without (-S9), the activation enzymes.

TABLE 3: SUMMARY OF IN VITRO MUTAGENICITY TEST RESULTS

Compound	Assay			
	Replicative/killing		Sister Chromatid Exchange	
	-S9	+S9	-S9	+S9
2-Acetylaminofluorene	-	+	-	+
Benzoin	-	?+	TBS	TBS
Caprolactam	-	-	TBS	TBS
Hexamethylphosphoramide	INC	INC	-	TBS
Safrole	?+	?+	TBS	TBS
O-Toluidine	-	-	-	TBS
2-Aminoanthracene	-	+	-	+
N-methyl-N-nitronitrosoguanidine	+	NT	+	NT

TBS To be scored.

INC Incomplete, a full test has not been conducted.

\+ Positive

\- Negative

?+ A weak positive response, requires confirmation at higher doses.

NT Not tested.

±S9 With or without metabolic activation enzymes.

Two of the chemicals for which results are given in Table 3 were not part of the International Program, but were added on our part to provide controls on the efficacy of the assays and the S9 response. N-methyl-N'-nitronitrosoguanidine (MNNG) is a direct-acting compound which should induce mutagenic changes in the absence of S9. This was confirmed in both assays. 2-aminoanthracene (2AA), on the other hand, requires S9 for the production of its mutagenically active intermediate. With both assays, 2AA gave a negative mutagenic response in the presence of S9. Thus, the S9 enzymes and the assays were responding normally.

Of the six other chemicals listed in Table 3, 2AAF, HMPA, safrole and O-toluidine would be expected to produce positive mutagenic data, primarily in the presence of S9, while benzoin and caprolactum should produce non-mutagenic responses. However, since the results are complete only for 2AAF, it is difficult to interpret accurately the response observed.

2AAF was positive in both assays in the presence of S9 and negative in both assays in the absence of S9, as was expected. Safrole produced a weak unconfirmed positive result on the RK assay. Since Safrole is known to be a potent carcinogen, a positive mutagenic result would be expected. On the other hand, O-toluidine was negative on the RK assay in the presence or absence of S9 and benzoin produced unconfirmed weak positive results in the presence of S9. Those results (if confirmed) may indicate that the RK assay does not discriminate well between positively and negatively mutagenic compounds. However, no current assay is completely

effective in such discrimination, and further comparative studies will be required to assess the effectiveness of these assay.

Because of the sparcity of data with the SCE assay, for which testing has only recently begun, it is difficult to identify any particular response pattern at this time.

This paper should be viewed as a progress report and only when final data are available from all the participating laboratories will more extensive conclusions be warranted in regard to both the chemicals tested and the utility of various assay systems.

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SUPPRESSION OF IMMUNE DEFENCES BY HALOGENATED AROMATIC HYDROCARBONS

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Introduction:

Industrialized communities, particularly in the region of the great lakes, recognize the potential threat to health posed by halogenated aromatic hydrocarbons in the environment. Polychlorinated biphenyls (PCBs) are endemic in southwestern Ontario and certain of the great lakes are significantly contaminated. A large reservoir of TCDD (2,3,7,8 tetrachlorodibenzo-p-dioxin) on Grand Island, New York, threatens Lake Ontario, and electric transformers and waste dumps elsewhere are of concern. The US Government resettled an entire community in a town where the roads had been sprayed with oil inadvertently contaminated with dioxin (TCDD) (1). It is estimated that 70-80 % of the population of Michigan harbours measurable levels of polybrominated biphenyls (PBBs) as a result of accidental contamination of cattle feed (2). Once any of these fat-soluble, poorly metabolized compounds enters the food chain, a wide variety of species may be subject to chronic exposure, including humans.

TCDD or dioxin (D1746016G), one of the most toxic of the halogenated aromatic hydrocarbons, has been the most extensively studied and provides a prototype model for haloaromatic toxicity. As illustrated in Table 1, toxic effects may be observed in a variety of organs (3,4). Susceptibility to toxicity requires the presence of a receptor protein to which TCDD binds in the cell (5-7). The level of the receptor is genetically controlled and binding of haloaromatic compounds such as PCBs and PBBs to the receptor is also associated with their ability to produce lesions similar to those that occur with dioxin (TCDD) (5-7). The major toxic effects recognized to date in humans exposed to TCDD (*) include skin lesions, liver damage and porphyria, neuropathy and neurasthenia, and hyperlipidemia (3,8). There is suggestive evidence for an increase in soft tissue sarcomas (9,10) and an increased tumor incidence has been seen in exposed animals (3). Considerable variation exists among different species of animals in susceptibility and pattern of tissue damage that develops following TCDD (3,4,11,12). Many species, for example, do not develop skin lesions. A common feature seen in all animal species which have been studied is atrophy of the thymus gland. Indeed, TCDD-treated guinea pigs that die rapidly after very low doses of TCDD show thymus atrophy as the main lesion.

TABLE I

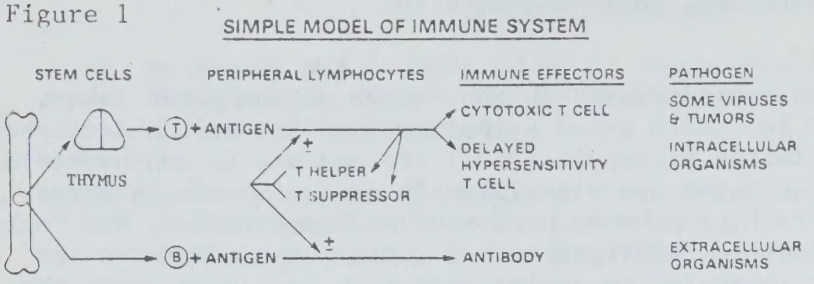
SPECTRUM OF LESIONS PRODUCED BY TCDD (HUMAN* AND ANIMAL)

Chloracne*, Pigmentation*, Hirsutism*
Liver damage* and phorphyria*
Neuropathy* and neurasthenia*
Hyperlipemia*
Embryotoxicity and malformations
Carcinomas and soft tissue sarcomas*?
Atrophy of thymus and increased sensitivity to bacterial toxins
Edema and pericardial effusion
Death

The thymus gland is a key organ in the immune defence system and produces at least four different types of blood lymphocytes (peripheral thymus-derived cells or T cells) that mediate resistance to infectious agents in the environment. As illustrated in figure 1, stem cells from bone marrow emigrate to thymus where they develop into cells that react to the alien (i.e. antigenic) determinants on foreign bacteria. On exposure to antigen, T cells undergo further development into different types of effector cells. Cytotoxic T cells (CTL) lyse virus infected cells and certain types of tumor cells associated with herpes-type viruses such as

the highly aggressive and fatal Kaposi's sarcoma seen in immunodeficient men and women (13,14). T cells which produce delayed hypersensitivity reactions (classical cell-mediated immunity) produce soluble mediators that activate macrophages. This activation is crucial if the macrophage is to deal effectively with certain types of intracellar agents (15) such as the tuberculosis and leprosy organisms. Activated macrophages may also play a role in resistance to some types of tumors (16). Two other types of T cells produced by the immune response serve to regulate the response by either increasing ("help") or inhibiting ("suppression") the production of effector cells (17). Stem cells from the bone marrow also develop independently of the thymus into antibody producing cells. Antibody is important in resistance to extracellar parasites. Since the antibody response is regulated by helper and suppressor T cells, however, a toxic effect of TCDD affecting production of helper and suppressor cells by the thymus could potentially affect all mechanisms of immune defence.

Figure 1



Over the past four years, we have conducted an extensive series of investigations in order to better understand what effect TCDD and related haloaromatic hydrocarbons might have on immunity and disease susceptibility. We have employed laboratory mice for these studies as their immune system is similar to that of man and genetically pure strains were available that differed in their susceptibility to TCDD (6,7). The objective of this paper is to summarize the results from these studies, to define problems requiring further investigation, and to outline insights gained concerning the problem of environmental exposure of humans to TCDD.

Type of immune dysfunction produced by TCDD in mice:

The design of the experiments is illustrated in figure 2. Following a series of 4 weekly doses of TCDD by injection, the animals were either challenged with antigen in vivo (as illustrated), or the cells removed from lymph nodes, spleen, and thymus for study in vitro. The in vivo assay system showed that the generation of cytotoxic T cells (CTL) was particularly sensitive to suppression by TCDD (Table II) and the mechanism of this effect was further analysed in vitro as shown in figure 3. Cell suspensions prepared from the lymph nodes, or spleen of normal and TCDD-treated mice, were cultured in test tubes with stimulator cells bearing the H-2^d alloantigen and the number

Figure 2

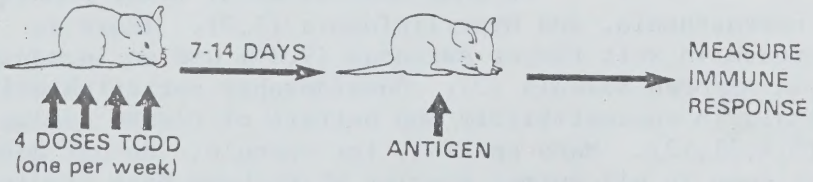
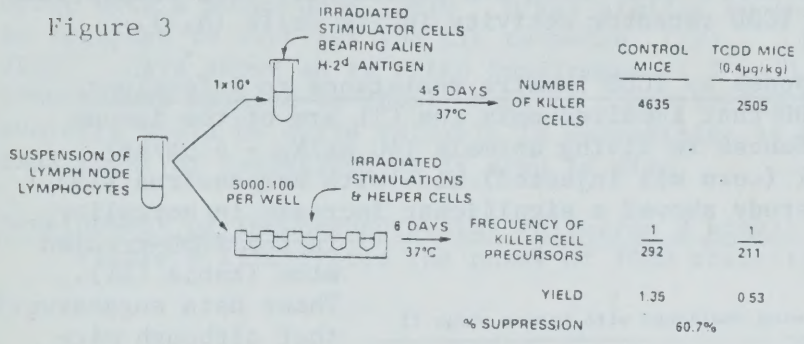


Table II

	DOSE OF TCDD (µg/kg over 4 weeks)				
	0.4	4	40	100	400
1. Appearance	Healthy	Healthy	Healthy	Sick	Sick
2. Cells in Lymphoid Organs					
Spleen & Lymph Nodes				↓	↓↓
Thymus		↓	↓↓	↓↓↓	↓↓↓
3. Type of Immune Response					
Antibody			↓↓ ±	ND	ND
Delayed Hypersensitivity		↓	↓	ND	ND
Cytotoxic T Cell	↓↓	↓↓	↓↓	ND	ND

of CTL produced against H-2^d was determined by measuring lysis of antigen-bearing target cells (% ^{51}Cr-release cytotoxicity assay). A typical result illustrated to the right in the upper panel of the figure shows that 10^6 lymph node cells from TCDD-treated mice produced only 2505 units of killer cells whereas the control mouse cells produced 4635 units. It was possible that the reduced CTL production by cells from TCDD-treated mice could have been caused by a toxic destruction of the precursors of CTL. However, in the lower panel of the figure, we actually measured the frequency of precursors by limiting dilution and as can be seen, there was in fact a slight increase in the frequency of CTL precursors (CTLP). We used these frequency measurements to calculate the number of precursors that had been added to the tube culture system and determined the yield (units of CTL activity generated per CTLP). This figure was reduced by 60.7 % in this experiment in animals treated with 0.4 µg/kg TCDD.

Figure 3

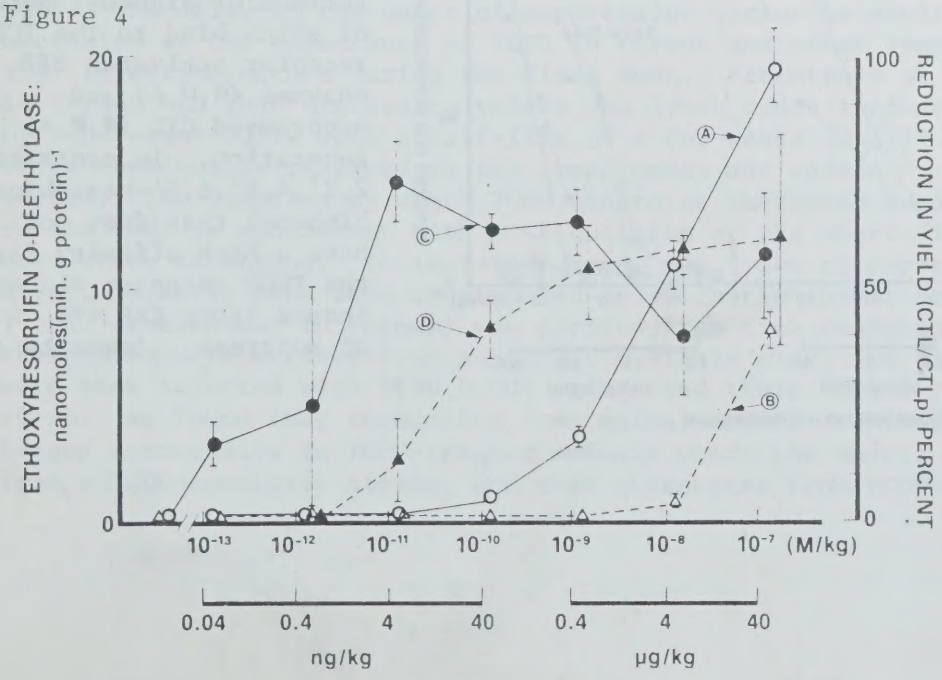


The reduction of CTL generation per precursor after TCDD exposure could have been due either to a lack of helper T cells or to an increase in suppressor T cells. Since the thymus appeared to be a key target organ for TCDD toxicity, we conducted some experiments to directly determine if TCDD treatment generated suppressor cells in the thymus. The result from this experiment has been published elsewhere (18). Briefly, we found that thymus cells from mice given a total TCDD dose of 0.4, 0.04, or 0.004 µg/kg (4 ng/kg) became suppressive. Suppression could not be attributed to TCDD itself that might have been present in the thymus. We therefore concluded that TCDD treatment generates suppressor T cells and it would seem from Table II that these suppressor cells act selectively to inhibit only the CTL response.

Dose of TCDD required to suppress immune defences:

A striking result consistently obtained in the study of TCDD-induced immune suppression was the impairment of immunity by as little as 0.004 mcg/kg (18-20). Since TCDD also activated hepatic mixed function oxidase enzymes, such as ethoxyresorufin-O-deethylase (ERR), we compared the dose of TCDD required to produce liver alterations to that which suppressed immunity. As shown in figure 4, the immune response of the peripheral lymph nodes TCDD-

Figure 4



sensitive C57Bl/6 male mice was suppressed with 4 ng/kg (C,●) whereas 10-100 X this dose was needed to produce a measurable rise in hepatic enzyme activity (D,▲). Similar observations were obtained in studies of the immune response (A,○) and hepatic ERR activity (B,△) of DBA/2 mice which are genetically less susceptible to TCDD by virtue of having less TCDD receptor activity in its cells (6,7).

Selective suppression of CTL response by TCDD impairs resistance to infection:

To determine if doses of TCDD that impaired only the CTL arm of the immune response also impaired immune defences in living animals (40 ng/kg - 4 µg/kg), we challenged treated and control (corn oil injected) mice with herpesvirus (19) type 2 i.p. The result of this study showed a significant increase in mortality in the TCDD-treated mice (table III). These data suggest that although mice exposed to ultra-low doses of TCDD appear healthy, they may suffer increased mortality if they are exposed to a type of pathogen such as herpesvirus where CTL appear to mediate immune resistance (14,21).

TABLE III

Effect of TCDD treatment on mortality following challenge with herpes virus II

Treatment	*Proportion surviving*	*Significance*[a]
Vehicle control[b]	33/76 (43.4%)	—
40 ng/kg TCDD	15/65 (23.0%)	0.009
0.4 µg/kg TCDD	18/64 (28.6%)	0.044
4.0 µg/kg TCDD	18/63 (29.6%)	0.051

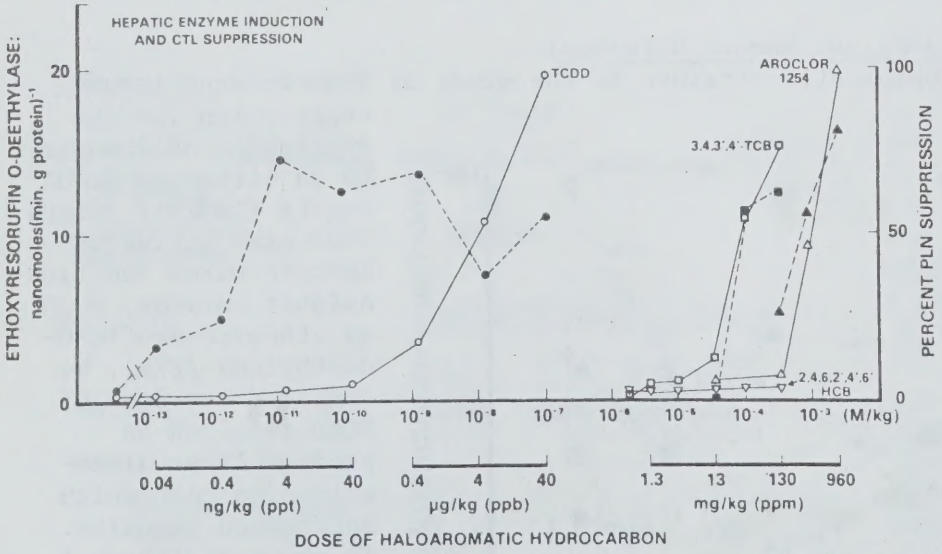
[a]P value in comparison to control was determined by Fisher's Exact test.

[b]Includes 16 untreated mice which had an identical survival to those receiving corn oil. All mice were male C57Bl/6J.

Other haloaromatic hydrocarbons that bind to TCDD receptor suppress immunity:

A variety of compounds in addition to TCDD may produce chloracne and other TCDD-like lesions; these haloaromatic hydrocarbons include PCVs, PBBs, polychlorinated dibenzofurans, and polychlorinated naphthalenes (4). A common feature of active hydrocarbons appears to be an ability to bind to the TCDD receptor. Therefore, we tested a variety of these non-TCDD haloaromatics for their effect on hepatic mixed function oxidase enzymes and for an effect on the CTL response in vitro. As illustrated in figure 5, Arochlor 1254, a mixture of PCBs, and 3,3',4,4'-tetrachlorobiphenyl both of which bind to the TCDD receptor activated ERR enzymes (○,□,△) and suppressed CTL (●,■,▲) generation. In contrast, 2,2',4,4',6,6'-hexachlorobiphenyl that does not have a high affinity for the TCDD receptor did not induce liver ERR (▽) or suppress immunity (19).

Figure 5

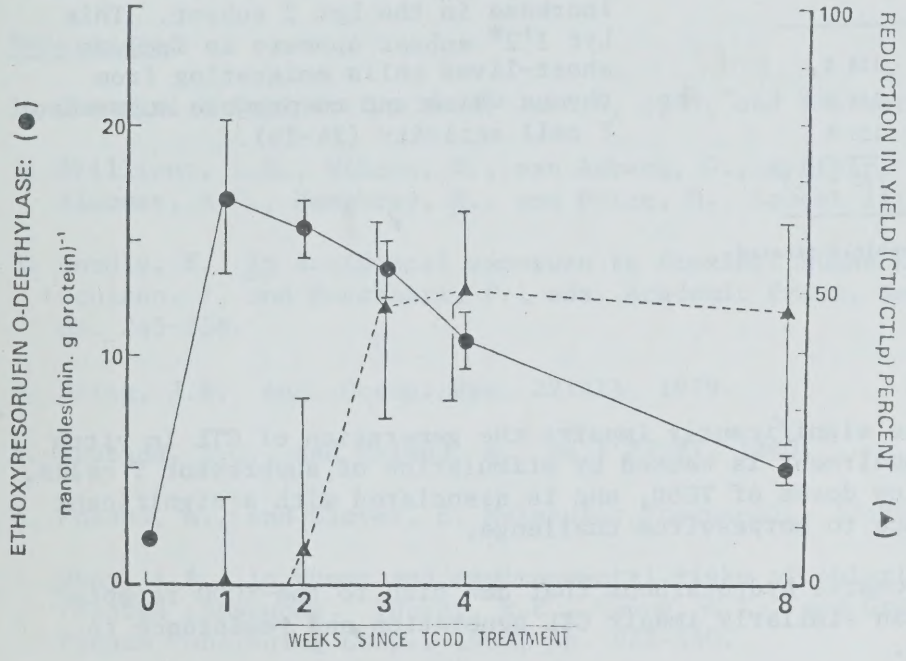


Imanishi et al (22) have reported that treatment of mice with the PCB mixture Kaneclor 500 significantly increases mortality following challenge with herpesvirus. Thus, PCBs may also produce the same defect in in vivo resistance to herpesvirus as we have shown with TCDD. The effect of the Kaneclor appears to occur with a molar dose ($3x10^{-6}$ M/kg) similar to the dose of 3,4,3',4'-TCB that is required to suppress the CTL response (19). Furthermore, Bekesi et al (23) have shown an in vitro impairment of T cell function in blood lymphocytes from humans exposed to PBBs. Data on the severity of viral infections in these subjects would be quite valuable in determining if humans incur a significant impairment of resistance as is seen in mice.

Development of immunosuppression following a single dose of TCDD:

Figure 6 illustrates the onset of TCDD toxicity as assessed by liver ERR. and CTL suppression. The mice were given a single injection of 0.4 µg/kg at time zero and were studied weekly thereafter for 4 weeks. It is readily apparent that hepatic ERR was rapidly induced and slowly declined in activity over an 8 week period. In contrast, immunosuppression was not evident until at least week 3 and persisted unchanged out to 8 weeks. Similar results were seen when 0.04 and 4 µg/kg TCDD were tested.

Figure 6



These data provided some clues concerning the mechanism of suppression by TCDD. The delay in the onset of suppression cannot be explained by a pharmacokinetic mechanism as the appearance of TCDD in thymus and other lymphoid organs following i.p. injection occurs during the first week. Precursors of T lymphocytes that mature in thymus and then migrate to spleen and lymph nodes include a short-lived population of suppressor cells with a half-life of a few weeks (24,25). This population is being continually replaced in the lymph nodes and spleen by new emigrants from the thymus. The kinetics by which TCDD generates increased suppressor T cell activity in the body is consistent with a stimulation of the short-lived suppressor T cell population in thymus. To test this idea, two types of experiments were conducted. First, chimeric mice were constructed in which the thymic stroma (which regulates T cell development in thymus) was derived from mice resistant to TCDD and the thymocytes were derived from susceptible strain mice, and vice versa. These animals were then injected with TCDD 0.004 µg/kg and their immune response studied in vitro. We found that thymocytes from animals DBA/2 resistant to this dose of TCDD became suppressive in TCDD-treated animals where the thymic epithelium was derived from a TCDD-sensitive strain, and that thymocytes from TCDD-susceptible C57Bl/6

mice failed to show increased suppressor cell activity when in the resistant DBA/2 strain host (20). The second type of study was conducted to enumerate the proportion of lymphocytes in PLN or spleen of TCDD-treated mice that possessed surface markers typical of short-lived suppressor T cells. In the mouse, these are called Lyt (lymphocyte) antigens and can be detected using an antibody to which a fluorescent label has been attached. We have used a cytofluograph which provides a sensitive and highly quantitative method for enumerating these cells, both in the mouse and in human blood, to test the effect of low dose TCDD treatment (4 ng/kg over 4 weeks) The result of 3 experiments in which murine lymph node cells were studied is illustrated in Table IV. The proportion of T cells bearing Lyt 2 was increased and further analysis indicted that the subset of Lyt 2 cells that also bear Lyt 1 was responsible for all of the increase in the Lyt 2 subset. This Lyt 1^+2^+ subset appears to include short-lived cells emigrating from thymus which act to promote suppressor T cell activity (24-26).

TABLE IV

Expt.	Lyt 1 only ("helper" T cells)	Ly 2 bearing
1	83.2 %[a]	118 %
2	85.0 %	121 %
3	105.0 %	135 %
	91.1 ± 7.0	125 ± 5.2[b]

a. TCDD as percent of result in vehicle-treated control.

b. Significant increase, $P < .05$

Summary and conclusions:

1. TCDD treatment of mice significantly impairs the generation of CTL in vitro and in vivo. This impairment is caused by stimulation of suppressor T cells, occurs at extremely low doses of TCDD, and is associated with a significant reduction in resistance to herpesvirus challenge.

2. Other types of haloaromatic hydrocarbons that can bind to the TCDD receptor protein in the cell can similarly impair CTL generation and resistance to herpesvirus challenge.

3. Although some strains of mice may be less susceptible to low dose TCDD, this resistance which is genetically determined by a gene or genes at the Ah locus is relative, and a sufficient dose of TCDD suppresses immune function of "resistant" animals.

4. Immunosuppression of mice with TCDD appears to increase the proportion of lymphocytes bearing the Lyt 2 marker that is found on suppressor T cells. Although a similar or analogous surface marker can be measured on suppressor T cells in human peripheral blood, it is not yet known if an increase in the number of suppressor cells is detectable in mouse blood. This information would be required before beginning a study of suppressor cells in haloaromatic hydrocarbon-exposed humans. Humans normally develop thymic involution during puberty so that the effects of TCDD and related compounds on the immune system might be expected to occur primarily in children and infants where the thymus is large and active.

5. The data obtained to date strongly implicates the epithelium of the thymus in the generation of suppressed immunity. Thymic epithelial cells elaborate a variety of regulatory molecules or thymic hormones (27) which control the immune system and assays now exist for some of these molecules. Measuring these thymic hormones in serum could provide a simpler and more sensitive test

for the immunotoxicity of TCDD and related haromatic hydrocarbons than measurement of suppressor cell number and activity in lymph nodes, spleen, or blood.

6. To date there has been little information concerning the possible effects of TCDD exposure on resistance to viral infections in humans apart from an increased frequency of complaints of colds (28) and it is possible that any immunological effects which occur following TCDD exposure may have minimal effect on resistance to most types of viruses. However, even small alterations in immunologic function indicate that the recipient has absorbed a sufficient amount of a haloaromatic hydrocarbon such as TCDD to incur a biological effect, and while the long term risk of such effects remains unknown at present their potential significance cannot be ignored.

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PARTITIONING OF MERCURY, LEAD AND CADMIUM IN AQUATIC SYSTEMS IN RELATION TO ACIDIFICATION

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1. Introduction

Acidification has the potential to influence the biogeochemical cycling of metals in aquatic systems. Recent concern over direct and indirect effects of acidic precipitation on surface waters has included consideration of potentially toxic trace metals. Some of these metals are "internally generated" from sediments, overburden or parent material, by increased weathering or dissolution from solid to dissolved phase as pH decreases. Al, Mn and Fe are in this category (1). Others are themselves trace contaminants which are loaded from external sources into the lake ecosystem and which may also increase in concentration as pH decreases (2). Cd and Pb are in this category. Zn would be in both categories.

Even if total dissolved metal concentration does not change, the chemical form or speciation of a number of metals in water or sediment is affected by pH. For example, Al changes solubility and dissolved species over the pH range 4-7, but Mn changes solubility but not speciation over the same range (3), while for Hg, there are speciation changes among methylated forms over this range (4).

Direct measurement of total or available metal in lakes of low alkalinity and depressed pH which are removed from

point sources of metals generally reveals low levels in the water, although Al and Mn are exceptions in this respect. In the absence of comprehensive sets of data on precipitation chemistry, dissolved metals and biological uptake, a predictive approach has been used to anticipate which metals are expected to be of concern as lakes acidify. Campbell et al. (1983) (5) evaluated 18 metals on the basis of the five criteria shown in Table 1. It can be seen that while a large number of metals are mobilised by man's activities, a relatively smaller number show pH-related changes in solubility and/or speciation with pH. It is those metals which are of potential concern for aquatic biota and ultimately for man. The latter 2 columns in Table 1 address toxicity and bioaccumulation, respectively. Uncertainty increases from left to right on the table, and more data on biological effects are urgently needed.

2. Biological effects of metals in acid lakes

Direct toxicity to biota is a possibility as metals increase in concentration in acidic lakes. In a recent survey of 36 lakes in Ontario, Stokes et al. (6) determined levels of dissolved metals and by principal components analysis ordinated the lakes according to certain groups of chemical properties. Dissolved metals were negatively correlated with the component determined by pH and alkalinity. Excluding point sources, (in this case the Sudbury region), only Al and Zn were present in concentrations likely to cause acute toxicity (Table 2). Cd

was determined in the water but was undetectable in all but 6 lakes. Al has of course been implicated already in a number of studies on fish toxicity in acid lakes (7,8), but to date no other metals have been shown to be related to changes in biota. But the effects of H^+ *per se* cannot be separated from correlated increases in metals without an experimental approach in the field or laboratory.

More information is available on the metal content of biota, i.e. bioconcentration or bioaccumulation, as distinct from toxicity. The phenomenon of biological uptake is well known though poorly understood for metal contaminated systems (9,10) and a number of workers have discussed the use of living organisms as biological monitors of available metals (11). Because organisms are believed to provide a means of determining the Available portion of total metal, and because they generally contain higher concentrations than the surrounding water, they provide a convenient means of monitoring metals which may be difficult to determine or speciate in water. At the same time, there is concern for the consequences of biological uptake because of the potential for food chain transfer and toxicity to high level consumers such as man. Bioaccumulation has been noted in acid lakes for at least 7 of the metals (Table 1) in lakes unaffected by point source contamination.

The balance of this paper reviews some of the available information on mercury, lead and cadmium.

3.1. Mercury

Aside from gross contamination from industrial sources, mercury is found in trace quantities in aquatic systems remote from any point sources (12). Although concentrations of Hg in water may be at the parts-per-trillion level, recent observations have indicated that fish tissues accumulate mercury from these low levels, and that for particular species of fish the concentrations in fish are higher in lakes of lower pH. This has been documented for example in yearling perch in Ontario (13) (Figure 1), for pike in Sweden (14), for walleye in Ontario (15), for pumpkinseed in Ontario (16) and for walleye in Wisconsin (17). One of the most satisfactory hypotheses has been put forward by Jernelov and Johansson (18) who propose that while microbial methylation of Hg is relatively unaffected by pH, the final product is predominately monomethyl mercury (the bioavailable form) at low pH, in contrast to dimethyl mercury at higher pH (Figure 2). This hypothesis has not been supported by experimentation in many different laboratories, but it still provides the simplest explanation to date. Other factors influencing mercury cycling and uptake by biota include higher productivity, variously considered to decrease mercury bioaccumulation by fish (19,20) or to increase it (21), suspended material such as clay (22), and fish growth rates (23).

Recent studies on filamentous algae demonstrated a significant relationship between mercury in algae and mercury in fish (24,25) (Figures 3 and 4). In one study,

the algal mercury was predominantly in the methyl form (24), supporting the contention that low pH increased the more available monomethyl form, since algae are not themselves known to methylate mercury. Recent data indicate the high levels of total mercury achieved by the algae (Table 3).

There are still technical problems with direct measurement of species of mercury in the low (2-20 ng/L) levels of total mercury typically found in water of acid-stressed lakes. However, a simple test of the hypothesis can be made in the laboratory. Earlier studies have measured direct uptake and food chain transfer of mercury in simple laboratory microcosms (26) but these workers were using much higher levels of mercury in water. We have designed experiments using methyl mercury in water without sediments, and measuring uptake at pH 6.8 and at 5.5 respectively, from initial concentrations of 50 and 100 ng/L. Organisms were selected on the basis of their occurrence in oligotrophic lakes over the selected pH range, their ease of handling and culturing in the laboratory and their acceptability as food.

After an extensive literature search, field sampling and laboratory trials, the amphipod Hyallela azteca was selected as primary consumer. It is an omniverous general scavenger in addition to being an important food source for many species of fish. It is found in acid-stressed lakes and in laboratory conditions shows mortality between pH 4 and 5 (27).

The selected food for Hyalella is Oocystis polymorpha;

as well the filamentous Mougeotia sp. isolated from an acidic lake will be used.

When direct uptake has been measured for both algae and for amphipods, and transfer from algae to amphipods via consumption has been determined, experiments with yearling perch will be carried out to complete the series.

3.2. Lead and cadmium

Although there is far less information available for lead and cadmium in biota of acid-stressed lakes, at least two separate studies have shown a relationship between levels of these metals in fish and pH of lakes (28,17). No such relationship was found in the survey of metals in algae (6); indeed for cadmium in particular most samples had undetectable cadmium and although algal lead was generally quite high, it was not possible to determine a relationship with lead in fish. A mechanism underlying the observed pH relationship with Cd or Pb has not been hypothesised but is unlikely to parallel that proposed for Hg.

A similar series of experiments is planned for partitioning of Cd and Pb in the model food chain described for Hg. Again, water concentrations will be realistically low, which is in contrast to other laboratory studies of metal uptake.

4. Summary

Using controlled experimental conditions, the effect of pH on the uptake, accumulation and food chain transfer of

Hg, Cd and Pb from water containing very low levels of the respective metals, will be determined. These experiments are designed to follow up correlations observed in field studies of metals in biota in acidic and neutral softwater lakes.

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TABLE 1

METALS OF CONCERN IN RELATION TO ACIDIFICATION OF SURFACE WATERS

METAL	ATMOSPHERE ANTHROPOGENIC CONTROL	INCREASED TOTAL CONCENTRATION	SPECIATION CHANGE CALC.	SPECIATION CHANGE OBS.	TOXICITY INH.	TOXICITY OBS. (IN ACID LAKES)	BIOACCUMULATION
AG	+				++		
AL		++	+	+	-	+	+
AS	+				+		
BE					-		
CD	+	+			++		+
CO	+			+	+		
CU	+		+		+		
HG	+	+	+	+	++		+
MN	+	++	+	+	-		+
MO							
NI	+				+		+
PB	+	+	+		+		+
SE	+				++		
SN					+		
TE							
TL					++		
V	+			+	++		
ZN	+	++		++	+		+

From Campbell et al., 1983. (Source, Reference 5)

TABLE 2

METALS	BACKGROUND[1] WATER LEVELS (PPB)	RANGE IN 1982 SURVEY STOKES ET AL.	TOXIC THRESHOLD (PPB)
ALUMINUM	30	46-372	100 (FISH)[2]
CADMIUM	0.07	1	0.2 (ZOOPLANKTON)[3]
COPPER	1.8	1-17	50 (ALGAE)[4]
LEAD	0.2	2-11	30 (ZOOPLANKTON)[5]
MANGANESE	5	5-276	-
NICKEL	0.3	2-6	30 (ZOOPLANKTON)[2]
ZINC	10	2-44	15 (ZOOPLANKTON)[3]

[1]FORSTNER AND WITMANN, 1981
[2]NRCC, 1981
[3]MARSHALL ET AL., 1981
[4]NRCC, 1979
[5]NRCC, 1978

(Source, Reference 6)

TABLE 3

TOTAL MERCURY IN ALGAE FROM SOFTWATER LAKES, 1983

LAKE	REGION	pH	TYPE OF GROWTH	TOTAL MERCURY $\mu g\ g^{-1}$ DRY WEIGHT
Tantaré	Laurentide Park, P.Q.	5.0	Natural (on macrophytes)	3.7
Least	Chapleau, Ontario	5.6	Artificial substrate	3.55
Horton	Chapleau, Ontario	6.4	Natural substrate	0.02
Chub	Dorset, Ontario	5.8	Artificial substrate	0.15
Lake 223	ELA	5.0	Natural (on macrophyte)	0.90
			Artificial	0.38

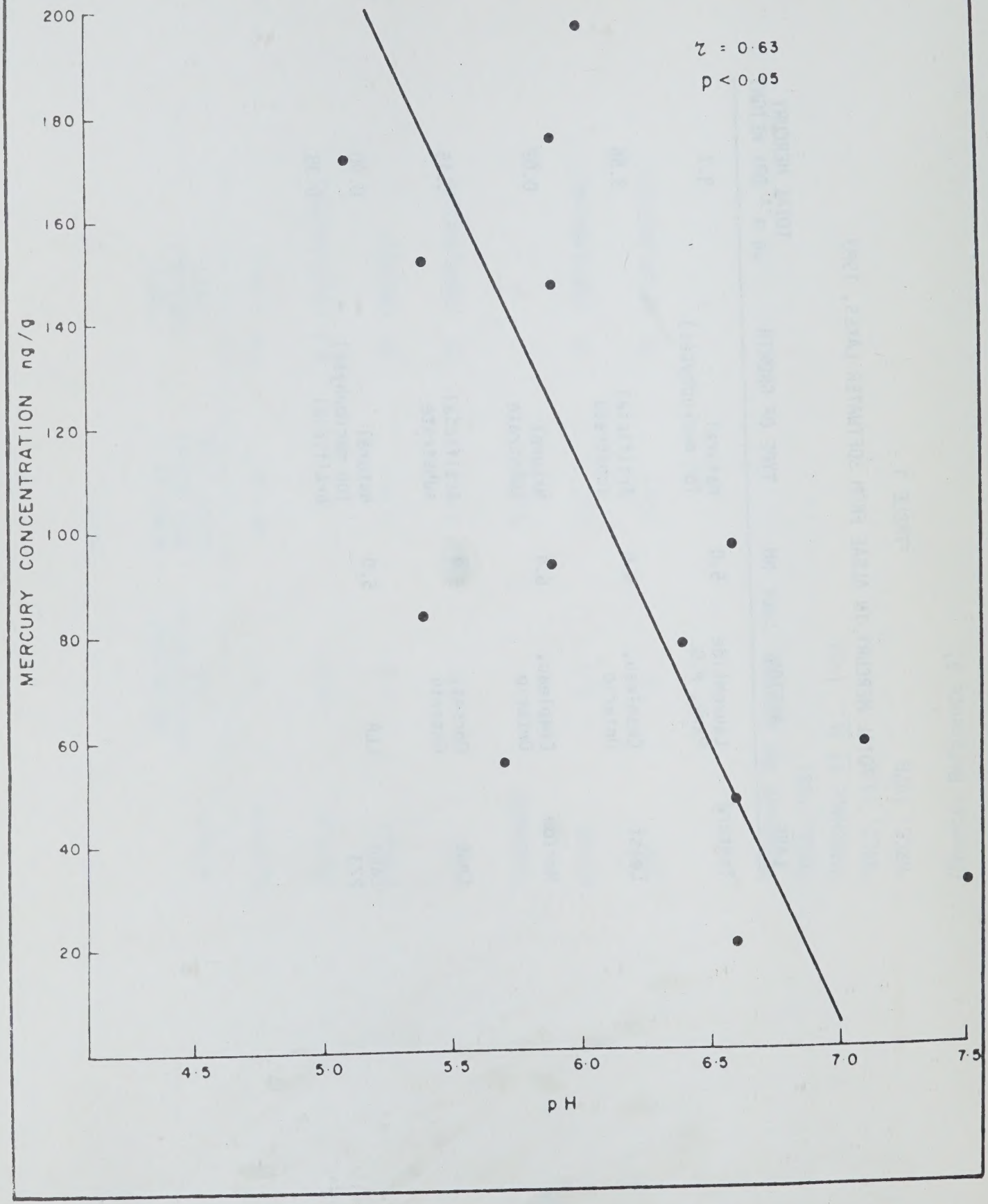

Figure 1: Mercury in yearling yellow perch and epilimnetic pH relationships. (Source, reference 13)

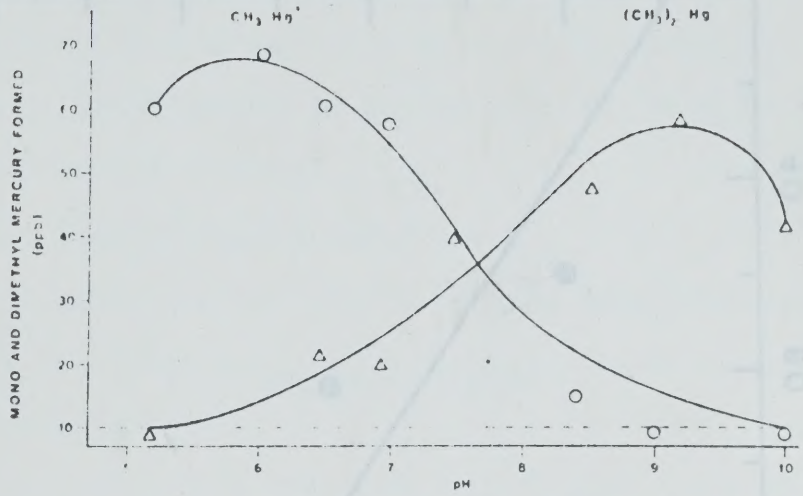

Figure 2: Formation of mono- and dimethyl mercury in organic sediments at different pH levels during 2 weeks, with a total mercury concentration of 100 ppm in substrate. (Source, reference 4)

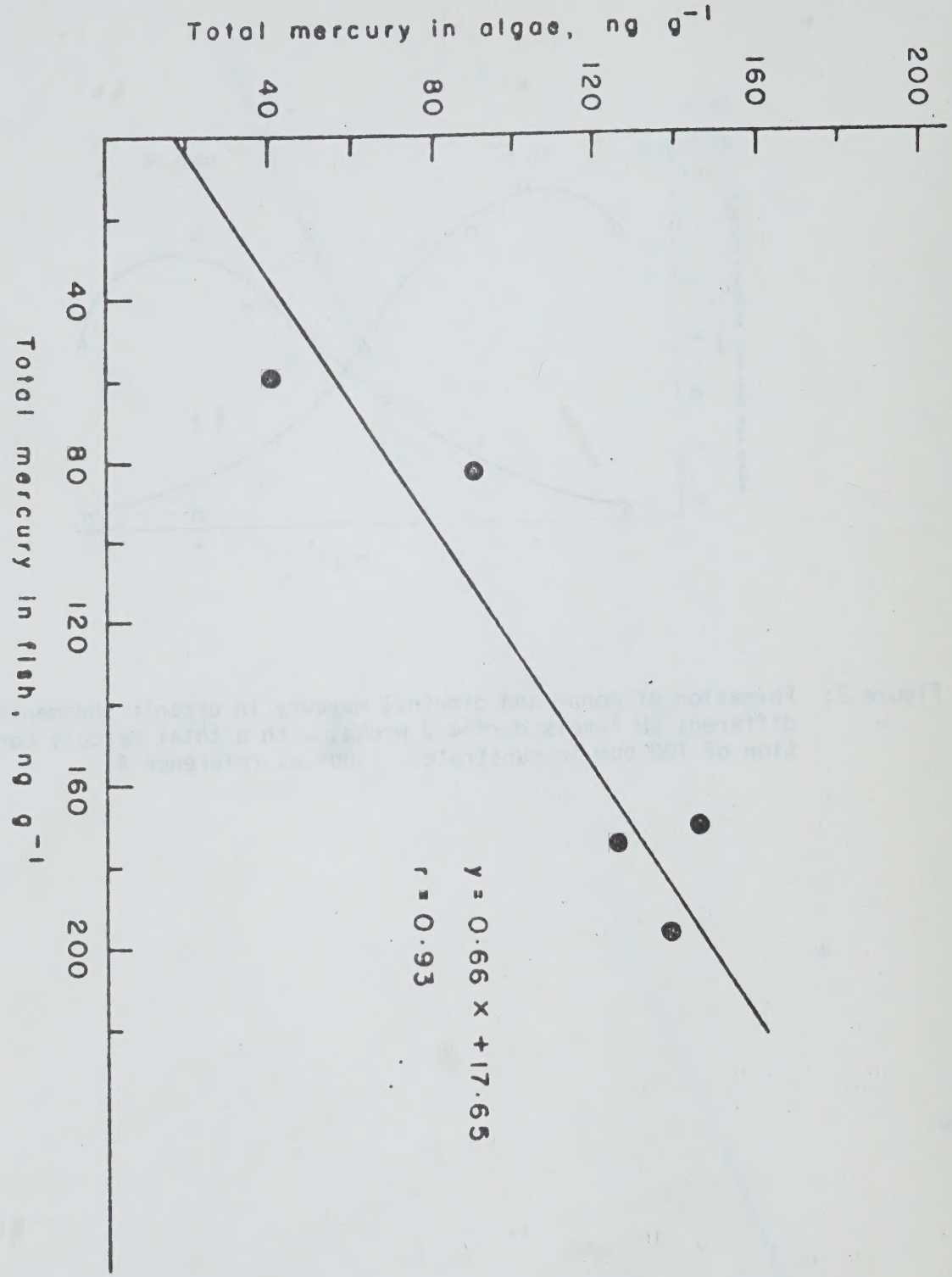

Figure 3: Relationship between mercury in yearling perch and mercury in algae, Dorset lakes, 1982. (Source, reference 24)

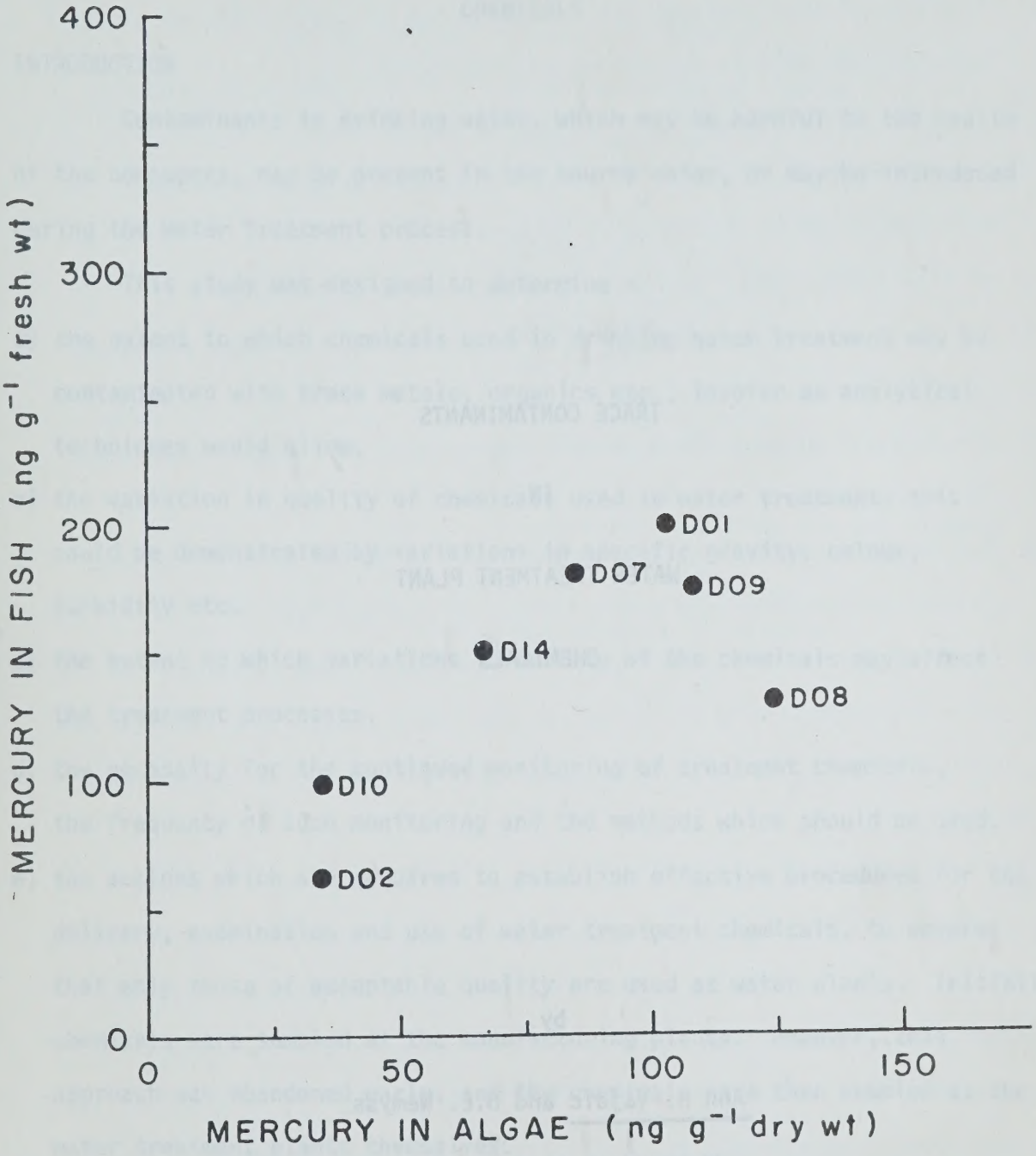

Figure 4: Relationship between mercury in yearling perch and mercury in algae, Dorset lakes, 1983. (Source, reference 25)

TRACE CONTAMINANTS IN WATER TREATMENT PLANT CHEMICALS

by

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TRACE CONTAMINANTS IN WATER TREATMENT PLANT CHEMICALS

INTRODUCTION

Contaminants in drinking water, which may be harmful to the health of the consumers, may be present in the source water, or may be introduced during the water treatment process.

This study was designed to determine -

a) the extent to which chemicals used in drinking water treatment may be contaminated with trace metals, organics etc., insofar as analytical techniques would allow.

b) the variation in quality of chemicals used in water treatment; this could be demonstrated by variations in specific gravity, colour, turbidity etc.

c) the extent to which variations in quality of the chemicals may affect the treatment processes.

d) the necessity for the continued monitoring of treatment chemicals, the frequency of such monitoring and the methods which should be used.

e) the actions which are required to establish effective procedures for the delivery, examination and use of water treatment chemicals, to ensure that only those of acceptable quality are used at water plants. Initially, chemicals were sampled at the manufacturing plants. However, this approach was abandoned early, and the chemicals were then sampled at the water treatment plants themselves.

This type of sampling programme had the added advantages of -

a) detecting contamination that might occur during transportation to the water plants, due to improper handling and/or storage, or the use of non-dedicated carriers eg. tanker trucks previously used for the transportation of other chemicals.

b) the samples taken would automatically sample more frequently the products of the manufacturers supplying the largest number of plants, and thus the largest quantities of chemicals.

The programme consisted of the following stages -

a) the development of a list of the most frequently used chemicals employed in water treatment in Ontario
b) an examination of production processes, where appropriate
c) the physical analysis of each chemical, where appropriate, including specific gravity, pH, colour and turbidity
d) the analysis of each chemical for trace metals and organic compounds, where possible
e) the development of broad recommendations for future monitoring, and further actions
f) the development of a guide for plant supervisors and operators for assessing chemicals upon arrival at treatment plants, and ensuring the safe, efficient handling and storage of those chemicals. (Appendix 1).

SAMPLING

Samples were categorized, as shown in Table 1, into primary coagulants, treatment aids (coagulant aids, water conditioners, filtration aids etc.), raw and treated waters, sludges and fluoridation chemicals. Sludge and treatment plant chemical samples were taken in wide-mouthed, acid-washed plastic bottles. Water samples were taken in 1L glass bottles; for metals other than mercury, 1L glass bottles were used preserved with 2 mL of 50% nitric acid per litre; for mercury 1 mL of concentrated nitric acid and 0.5 mL drops of saturated potassium dichromate solution was added to samples taken in bacteriological sample bottles according to laboratory specifications. (1). The water plants at which the treatment chemicals were sampled are shown in Table 2.

The water plants were selected on the basis of the following criteria -

TABLE 1

WATER TREATMENT CHEMICAL CATEGORIES

PRIMARY COAGULANTS -	Aluminum sulphate - solid Aluminum sulphate - liquid Ferric chloride Sodium aluminate
TREATMENT AIDS -	Calcium oxide (unslaked lime) Sodium bicarbonate Sodium carbonate (soda ash) Sodium hydroxide Activated carbon Cat Floc T
WATER -	Raw, untreated water Finished, treated water
SLUDGE -	Usually a mixture of old sludge, from settling basins and new sludge, from settled backwashes
FLUORIDATION CHEMICALS -	Sodium silicofluoride Hydrofluorosilicic acid

TABLE 2

Water Plants Sampled for Treatment Chemicals

Plant	Location	Source of Supply	Capacity $(1000m)^3$
Stage 2			
Caldwell W.S.*	Verner	Veuve River	1.059
R.C. Harris WTP**	Toronto	Lake Ontario	1000.12
R.L. Clark WTP	Toronto	Lake Ontario	454.6
Stage 3			
Elgin Area W.S.	Yarmouth Twp.	Lake Erie	45.5
R.H. Neath WTP	Owen Sound	Georgian Bay	30.004
Sarnia City W.S.	Sarnia	Lake Huron	181.84
Goderich WTP	Goderich	Lake Huron	12.001
Port Elgin WTP	Port Elgin	Lake Huron	8.706
R.L. Clarke WTP	Toronto	Lake Ontario	454.6
Grand Bend Supply	Grand Bend	Lake Huron	168.2
Stage 4			
Easterly WP	Scarborough	Lake Ontario	454.6
Lindsay WTP	Lindsay	Scugog River	22.730
Kenora WTP	Kenora	Lake of the Woods	26.594
R.C. Harris WTP	Toronto	Lake Ontario	1000.12
Arnprior WTP	Arnprior	Madawaska River	15.456
Cornwall WTP	Cornwall	Lake St. Lawrence	100.012
Hawkesbury	Hawkesbury	Ottawa River	18.184
Ottawa WSS	Brittania	Ottawa River	247.302

*WS - water system

**WTP - water treatment plant

a) they received treatment chemicals from a good cross-section of the chemical manufacturers.

b) they had large flow rates. The greater the volume of water treated, the quicker the turn-over of chemicals and therefore the fresher the chemicals tested.

c) they were distributed as evenly as possible across the Regions and represented both lake and river supplies.

ANALYTICAL METHODS

A. Physical

Colour and turbidity were examined visually and measured photometrically with a Hach DR2 spectrophotometer and any gross foreign contamination was noted.

The pH was determined electrometrically, directly and in 1% solutions prepared in distilled water, on a Fisher Accumet pH meter. Specific gravity was determined by weighing on a Sauter balance, that had been checked for accuracy. Electron microscope studies were carried out using a Siemans 102 model, transmission electron microscope.

B. Chemical

Analysis for trace metals and organic compounds was carried out using standard preparatory techniques, followed by measurement by atomic absorption, emission spectography, gas chromatography and spectrophotometric colorimetry. Analyses were generally carried according to the methods prescribed in the "Outlines of Analytical Methods". (1). Some ad hoc modifications were required, due to the nature of some of the samples, along with the use of special standards.

Some types of sample could not be analysed because of severe interference or a highly corrosive effect on laboratory equipment. Other analysis produced results with very wide confidence limits, or of the

"less than" variety, which were essentially qualitative. Chlorine was not able to be analysed for technical reasons, as was the case with many of the polyelectrolyte however, the former is generally used at dosages of less than 5 ppm and the latter at less than 1 ppm.

PROGRAMME

In the initial stages, plant chemicals and raw and treated waters and water plant sludges from various water plants, were analysed. Analysis for thirty trace metals, organics by solvent extraction/gas chromatography as well as for physical properties was completed. As the programme progressed, it became evident that some analyses were not going to be successful for some types of sample, and they were abandoned. For example, electron microscope analysis of primary coagulants was terminated because a suitable method could not be developed; solvent extraction and gas chromatography analysis was not done for the raw and treated water samples or the treatment aids because initial results had been consistently negative. Analysis for trace metals was changed from emission spectrography to atomic absorption to obtain more accurate results.

The programme was thus a dynamic one, with changes being made, as appropriate, when results were received in order to obtain the most accurate results. The analyses carried out in the programme are shown in Chart A.

RESULTS

In order to assess the effects of the various contaminants in drinking water chemicals on the treatment process and the quality of the finished water, it is most useful to discuss selected results collated from the whole programme. In this way, a total picture of quality variation in treatment chemicals both with respect to contamination levels and physical properties can be obtained.

CHART A

	Analyses	Primary Coagulants	Treatment Aids	WTP Raw/Treated Waters	WTP Sludge	Fluoridation Chemicals
STAGE 1	Trace Metals	List A1				
	Physical Properties	List B1				
	Solvent Extraction/ Gas Chromatography					List C
	Electron Microscope	List D				
STAGE 2	Trace Metals	List A2		List A2	List A2	
	Physical Properties	List B1				
	Solvent Extraction/ Gas Chromatography	List C		List C	List C	List C
	Electron Microscope	List D				
STAGE 3	Trace Metals	List A2	List A2	List A2	List A2	List A2
	Physical Properties	List B1				
	Solvent Extraction/ Gas Chromatography	List C			List C	List C
	Electron Microscope			List D		
	General Chemical			List E		
	Carbon Analyser	List F	List F	List F	List F	
	Mercury			List G		
	Loss on Ignition	List H2	List H2		List H2	
	Solvent Extraction/IR	List J	List J		List J	
STAGE 4	Trace Metals	List A2	List A2	List A2	List A2	
	Physical Properties	List B2				
	Solvent Extraction/ Gas Chromatography	List C				List C
	Electron Microscope					
	General Chemical			List E		
	Carbon Analyser	List F	List F	List F	List F	
	Mercury	List G	List G	List G		
	Loss on Ignition	List H1			List H2	
	Solvent Extraction/IR	List J	List J		List J	

List A

Trace Metals

A1
- Cadmium
- Chromium
- Copper
- Nickel
- Lead
- Manganese
- Zinc
- Cobalt
- Tin
- Molybdenum

A2
- Barium
- Mercury
- Iron
- Silver
- Aluminum
- Arsenic
- Beryllium
- Bismuth
- Boron
- Zirconium
- Calcium
- Gallium
- Germanium
- Indium
- Magnesium
- Tellurium
- Silicon
- Antimony
- Strontium
- Titanium
- Vanadium

List B

Physical Properties

B1
- Specific Gravity
- Turbidity
- pH

B2
- % Alumina
- % Free Acid
- Colour/Turbidity

List C

Solvent Extraction/
Gas Chromatography

- 2-4 DNT
- 2-6 DNT
- TND
- Other N Compounds

List D

Electron Microscope

Asbestos Fibres &
Other Particles

List E

General Chemical

- pH
- Alkalinity
- Hardness
- Chlorine
- Iron
- Turbidity

List F

Carbon Analyser

Dissolved Carbon

List G

Mercury

Hg

Flameless Atomic Abs.

List H

Loss on Ignition

H1
- % H20

H2
- % Loss on Ignition
- Total Carbon

List J

Solvent Extraction

Methylene Chloride Extraction

Infra Red Spectrophotometry

TRACE METAL CONTAMINATION

Table 3 shows the recommended limits or maximum acceptable concentration (MAC) for selected trace metals from the Ontario Drinking Water Objectives (2); further, the table shows the concentration of metal that would have to be present in the drinking water chemicals to result in the MAC appearing in the finished drinking water, assuming a dose of 50 ppm, 25 ppm or 5 ppm of the chemical has been added for treatment. For example, for a chemical used at a dose of 50 ppm, 1000 ppm of arsenic would have to be present in that chemical to produce the MAC (0.05 ppm) in the finished water; at a dose of 25 ppm, then 2000 ppm of contaminating arsenic would be required and at 5 ppm, the required arsenic level would be 10,000 ppm. In the following discussion, the four most toxic metals will be considered. Since there was a good deal of interference with the analytical techniques, the accuracy of many of the results was equivocal, being of the "less than" variety or having wide confidence limits.

Some typical results of trace metal analysis in primary coagulants are shown in Table 4. As can be seen, one sample of liquid alum showed manganese, iron, titanium and vanadium levels considerably higher (more than five times) than the other samples; two samples showed chromium levels higher and one showed a lead level of 96 ppm as opposed to less than 1 ppm for the other two samples. The samples of solid alum showed similar trends, with sample 1 showing elevated metal levels. In primary coagulants the highest definitive level of arsenic recorded was 0.08 ppm, the highest for cadmium was 0.67 ppm, for lead 96 ppm and mercury 0.05 ppm; the equivalent highest recorded levels were less than 300 ppm, less than 80 ppm, less than 80 ppm and less than 300 ppm respectively. These levels may be plotted on a chart and compared to those levels (from Table 3), required to produce the MAC in the finished water. Such a plot is shown in Chart 1, assuming the coagulants are used at a dose of 50 ppm.

TABLE 3. RECOMMENDED LIMITS FOR METALS IN DRINKING WATER AND CONTAMINATION LEVELS REQUIRED IN TREATMENT PLANT CHEMICALS TO REACH THOSE LIMITS, ASSUMING THE SPECIFIED DOSAGES.

Metal	Recommended Limits	Concentrations of contaminant in treatment chemical that is required to donate the MAC at the following dosages		
	MAC*(mg/L)	50 ppm	25ppm	5 ppm
Arsenic	0.05	1,000	2,000	10,000
Barium	1.0	20,000	40,000	200,000
Boron	5.0	100,000	200,000	1,000,000
Cadmium	0.005	100	200	1,000
Lead	0.05	1,000	2,000	10,000
Mercury	0.001	20	40	200
Selenium	0.01	200	400	2,000
Silver	0.05	1,000	2,000	10,000
Uranium	0.02	400	800	4,000
Chromium	0.05	1,000	2,000	10,000

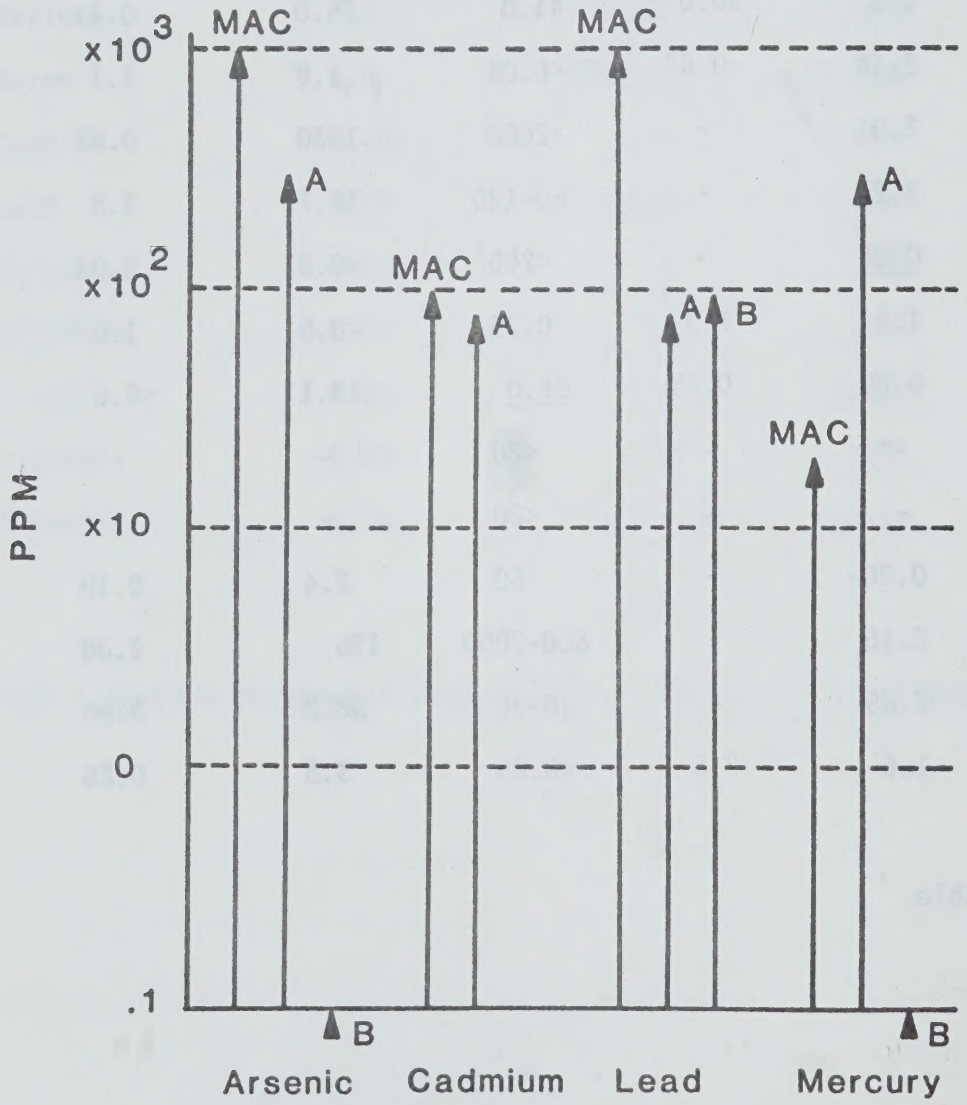
*MAC = maximum acceptable concentration, Ontario Drinking Water Objectives

TABLE 4. TYPICAL TRACE METAL RESULTS OF PRIMARY COAGULANTS.

Trace Metal (ppm) as metal	COAGULANT Liquid Alum(1)	Liquid Alum(2)	Liquid Alum(3)	Solid Alum(1)	Solid Alum(2)	Ferric Chloride
Arsenic	-	-	0.08	-	-	<300
Beryllium	<0.03	-	<2	<0.03	<0.03	<10
Cadmium	<0.05	0.67	0.03	<0.05	<0.05	<10
Cobalt	<0.5	0.52	<0.2	<0.5	<0.5	10-30
Chromium	1.8	50.0	41.0	75.0	0.41	~10
Copper	2.14	0.67	<0.05	1.8	1.7	10-30
Iron	2.91	-	>2000	1530	0.82	-
Manganese	3.25	-	60-120	12.7	3.8	-
Mercury	0.05	-	<200	<0.01	0.03	0.05
Nickel	1.5	1.3	0.35	<0.5	1.0	10-30
Lead	0.88	0.25	96.0	13.1	<0.6	<10
Antimony	-*	-	<20	-	-	<30
Tin	-	-	<20	-	-	<30
Strontium	0.28	-	<60	2.4	0.18	<100
Titanium	2.15	-	600-2000	136	3.08	-
Vanadium	2.85	-	10-30	38.2	3.86	10-30
Zinc	1.6	2.6	<0.25	3.5	0.25	-

*no result available

CHART 1. COMPARISON OF THE HIGHEST LEVELS OF FOUR TOXIC METALS (ARSENIC, CADMIUM, LEAD and MERCURY) REPORTED IN PRIMARY COAGULANTS, WITH THE LEVELS REQUIRED TO PRODUCE THE MAC* IN THE FINISHED WATER, ASSUMING A DOSE OF 50ppm.



* MAC - Maximum acceptable concentration in ODWO. Levels in excess of the MAC are of health concern.

A - High Level

B - High Definitive Level

With the exception of mercury, none of the levels of metals found exceeded that required to produce the MAC in the finished water. With mercury, only the "less than" 300 ppm measurement, which is not considered reliable, is beyond the critical level.

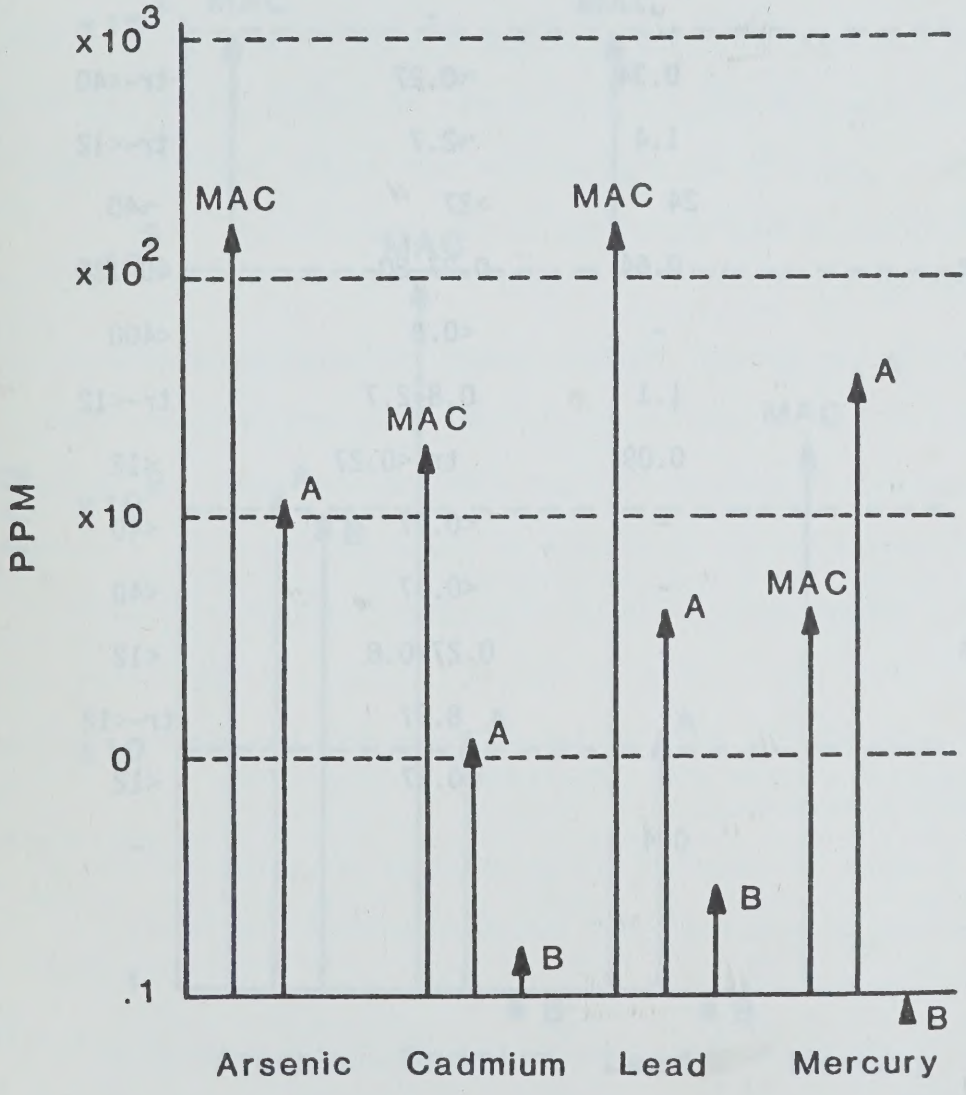
Table 5 shows typical metal analysis for the treatment aids; as can be seen, there was some variation in metal levels. Since these are generally used at lower dose rates than the primary coagulants, a plot similar to that developed for the primary coagulants was made, but using a dose rate of 25 ppm. The highest level found for arsenic was less than 120 ppm in unslaked lime and Cat-Floc T and there was no more accurately determined maximum. For cadmium, the highest level was less than 12 ppm in Cat Floc T and the highest definitive level, 1.6 ppm in unslaked lime. Lead showed corresponding levels of about 40 ppm in Cat Floc T and 2.9 ppm in unslaked lime, whereas mercury showed levels of less than 400 ppm in Cat Floc T and unslaked lime and no more accurately determined maximum; levels determined by atomic absorption spectrophotometry were <0.01 ppm. These levels are plotted in Chart 2. The fluoridation chemicals are usually used at a dose of about 5 ppm. Table 6 lists typical trace metal results for these chemicals. The highest level of arsenic recorded was less than 120 ppm in a sample of sodium silicofluoride, with a definitive level of 97 ppm being found in a sample of fluorosilicic acid.

For cadmium the highest level was less than 12 ppm in sodium silicofluoride with a level of 0.02 ppm in two samples of hydrofluorosilicic acid. Equivalent numbers for lead were less than 12 ppm in sodium silicofluoride and 0.09 ppm in hydrofluorosilicic acid, and for mercury, less than 400 ppm, in sodium silicofluoride with no accurately determined figure. As can be seen, however, from the plots in Chart 3, none of these levels except the questionable <400 ppm result for mercury would result in the MAC appearing

TABLE 5. TYPICAL TRACE METAL RESULTS OF VARIOUS TREATMENT AIDS.

Trace Metal (ppm) as Metal	TREATMENT AID				
	Activated Carbon	Unslaked Lime	Polymer Cat-Floc T	Sodium Bicarbonate	Sodium Hydroxide
Arsenic	<0.2	<120	<120	-	<100
Beryllium	<0.5	-	<12	<0.6	<0.5
Cadmium	0.1	1.6	<12	<0.05	<10
Cobalt	-	<1.0	-	<0.5	<1.0
Chromium	2.5	23.0	<12	0.12	tr <0.5
Copper	0.9	12.0	~12	<0.6	0.5-1.0
Iron	540.0	~4000	1200-4000	<0.36	10-20
Manganese	16.0	40-120	tr-<12	<0.6	tr-0.5
Mercury	-	<400	<400	<0.01	-
Nickel	3.1	2.6	tr-<12	<0.5	<3
Lead	<0.4	2.9	~40	<0.6	<10
Antimony	-	<40	<400	-	<50
Tin	-	<40	<12	-	<10
Strontium	2.4	400-1200	<120	2.05	3-10
Titanium	8.0	12-40	<12	<1.25	<0.5
Vanadium	<2.0	tr-<12.0	<12	<1.25	-
Zinc	0.78	9.3	-	0.88	<10

CHART 2. COMPARISON OF THE HIGHEST LEVELS OF FOUR TOXIC METALS (ARSENIC, CADMIUM, LEAD and MERCURY) REPORTED IN TREATMENT AIDS, WITH THE LEVELS REQUIRED TO PRODUCE THE MAC* IN THE FINISHED WATER, ASSUMING A DOSE OF 25ppm.



* MAC - Maximum acceptable concentration in ODWO. Levels in excess of the MAC are of health concern.

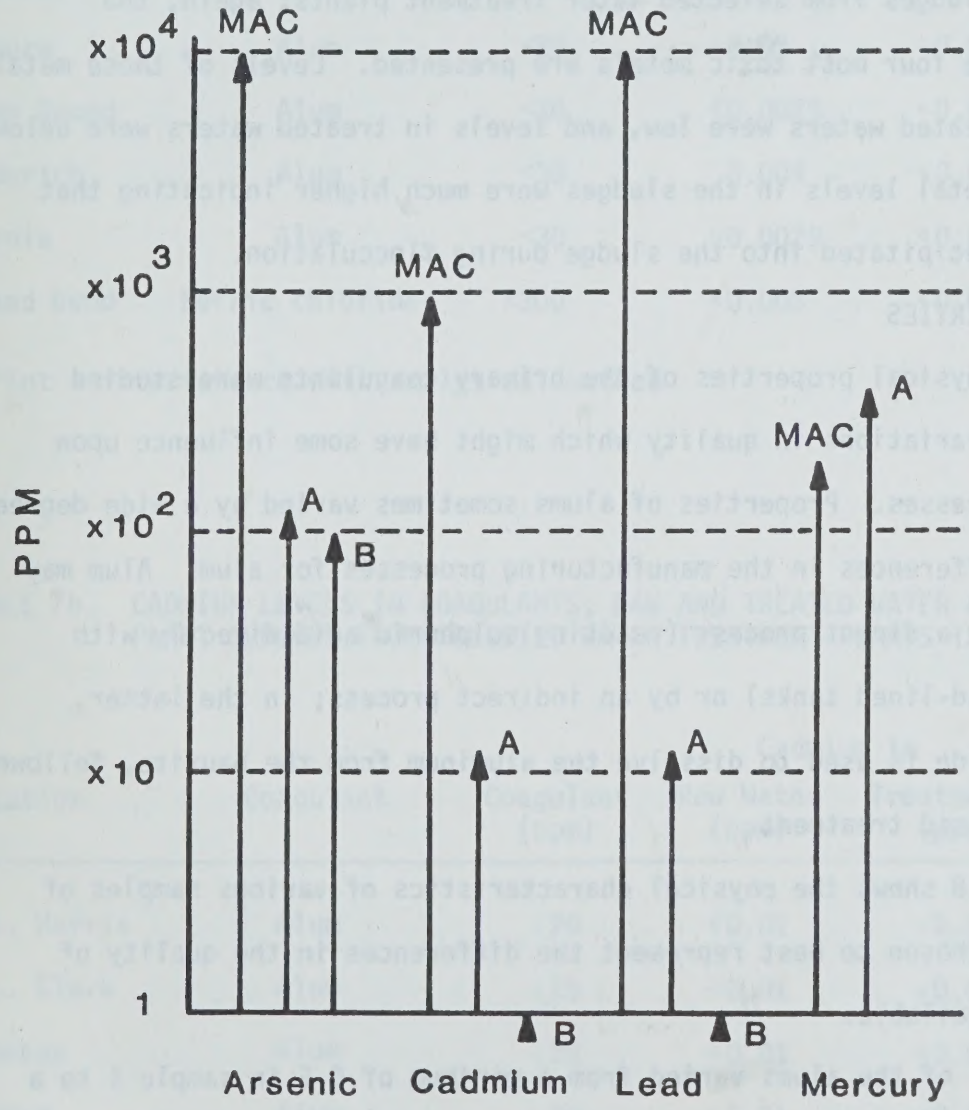
A - High Level

B - High Definitive Level

TABLE 6. TYPICAL TRACE METAL RESULTS OF FLUORIDATION CHEMICALS

Trace Metal (ppm) as Metal	CHEMICAL		
	Hydrofluorosilicic Acid		Sodium Silicofluoride
	1	2	
Arsenic	97.0	∿0.8	<120
Beryllium	-	<0.27	<12
Cadmium	0.02	<0.27	<12
Cobalt	-	-	-
Chromium	0.34	∿0.27	tr-<40
Copper	1.4	∿2.7	tr-<12
Iron	24	>27	∿40
Manganese	0.64	0.27-80	40-120
Mercury	-	<0.8	<400
Nickel	1.1	0.8-2.7	tr-<12
Lead	0.09	tr-<0.27	<12
Antimony	-	<0.27	<40
Tin	-	<0.27	<40
Strontium	-	0.27-0.8	<12
Titanium	-	8.27	tr-<12
Vanadium	-	∿0.27	<12
Zinc	0.4	-	-

CHART 3. COMPARISON OF THE HIGHEST LEVELS OF FOUR TOXIC METALS (ARSENIC, CADMIUM, LEAD and MERCURY) REPORTED IN FLUORIDATION CHEMICALS, WITH THE LEVELS REQUIRED TO PRODUCE THE MAC* IN THE FINISHED WATER, ASSUMING A DOSE OF 5ppm.



* MAC - Maximum acceptable concentration in ODWO. Levels in excess of the MAC are of health concern.

A - High Level

B - High Definitive Level

in the finished water if the chemicals were used at their usual dose rate.

As can be seen, the levels of these toxic metals found contaminating water treatment chemicals, are, for the most part, significantly below the levels which would result in a health concern in the finished water, even if used at abnormally high dose rates. Tables 7a, b, c and d, present the results of analysis of coagulant chemicals, raw and treated waters and water plant sludges from selected water treatment plants; again, the results of the four most toxic metals are presented. Levels of these metals in raw and treated waters were low, and levels in treated waters were below the MAC's. Metal levels in the sludges were much higher indicating that metals are precipitated into the sludge during flocculation.

PHYSICAL PROPERTIES

The physical properties of the primary coagulants were studied to determine variations in quality which might have some influence upon treatment processes. Properties of alums sometimes varied by a wide degree, due to the differences in the manufacturing processes for alum. Alum may be produced by a direct process (reacting sulphuric acid directly with bauxite in lead-lined tanks) or by an indirect process; in the latter, sodium hydroxide is used to dissolve the aluminum from the bauxite, followed by sulphuric acid treatment.

Table 8 shows the physical characteristics of various samples of liquid alum, chosen to best represent the differences in the quality of liquid alum available.

The pH of the alums varied from a minimum of 0.5 in sample 4 to a maximum of 2.5 in sample 6; the free acid occurred at a high of 1.23% in sample 4 and free alumina occurred at a high of 0.28% in sample 6.

Differences in the pH of alums could potentially affect the functioning of the chemical in treatment. As shown in Table 9, water with a moderate buffering capacity (100 ppm alkalinity) suffers a maximum pH reduction of

TABLE 7a. ARSENIC LEVELS IN COAGULANTS, RAW AND TREATED WATER AND WATER PLANT SLUDGES FROM SELECTED WATER TREATMENT PLANTS IN ONTARIO

Location	Coagulant	Arsenic in Coagulant (ppm)	Raw Water (ppm)	Treated Water (ppm)	Sludge (ppm)
R.C. Harris	Alum	<20	<0.05	<0.05	0.88
R.L. Clark	Alum	<20	<0.05	<0.05	0.73
Lindsay	Alum	<20	<0.05	<0.05	0.32
Kenora	Alum	<20	<0.05	<0.05	0.37
Owen Sound	Alum	<30	<0.0025	<0.0025	0.26
Goderich	Alum	<30	0.004	<0.0025	Int*
Sarnia	Alum	<30	<0.0025	<0.0025	Int*
Grand Bend	Ferric chloride	<300	<0.003	<0.0026	Int*

*Int = interference with analytical method

TABLE 7b. CADMIUM LEVELS IN COAGULANTS, RAW AND TREATED WATER AND WATER PLANT SLUDGES FROM SELECTED WATER TREATMENT PLANTS IN ONTARIO

Location	Coagulant	Cadmium in Coagulant (ppm)	Raw Water (ppm)	Treated Water (ppm)	Sludge (ppm)
R.C. Harris	Alum	<20	<0.01	<0.01	0.19
R.L. Clark	Alum	<20	<0.01	<0.01	<0.02
Lindsay	Alum	<20	<0.01	<0.01	<0.02
Kenora	Alum	<20	<0.01	<0.01	<0.02
Owen Sound	Alum	<10	<0.0005	<0.0005	0.51
Goderich	Alum	<10	<0.0005	<0.0005	<0.02
Sarnia	Alum	<10	<0.0005	<0.0005	0.1
Grand Bend	Ferric chloride	<10	0.0005	0.0005	0.6

TABLE 7c. LEAD LEVELS IN COAGULANTS, RAW AND TREATED WATER AND WATER PLANT SLUDGES FROM SELECTED WATER TREATMENT PLANTS IN ONTARIO

Location	Coagulant	Lead in Coagulant (ppm)	Raw Water (ppm)	Treated Water (ppm)	Sludge (ppm)
R.C. Harris	Alum	<10	<0.05	<0.05	<0.4
R.L. Clark	Alum	<20	<0.05	<0.05	1.4
Lindsay	Alum	<20	<0.05	<0.05	<0.4
Kenora	Alum	<20	<0.05	<0.05	<0.4
Owen Sound	Alum	<10	0.004	0.007	<0.4
Goderich	Alum	<10	0.0095	0.006	5.6
Sarnia	Alum	<10	<0.0025	<0.0025	<0.4
Grand Bend	Ferric chloride	<10	0.007	0.009	14.7

TABLE 7d. MERCURY LEVELS IN COAGULANTS, RAW AND TREATED WATER AND WATER PLANT SLUDGES FROM SELECTED WATER TREATMENT PLANTS IN ONTARIO

Location	Coagulant	Mercury in Coagulant (ppm)	Raw Water (ppm)	Treated Water (ppm)	Sludge (ppm)
R.C. Harris	Alum	<200	<0.00008	<0.00008	-
R.L. Clark	Alum	<200	0.00005	0.00005	-
Lindsay	Alum	<200	<0.00003	<0.00003	-
Kenora	Alum	<200	0.00005	0.00007	-
Owen Sound	Alum	<100	<0.00003	<0.00003	-
Goderich	Alum	<100	<0.00004	<0.00004	-
Sarnia	Alum	<100	<0.00004	<0.00004	-
Grand Bend	Ferric chloride	<300	<0.00004	<0.00004	-

TABLE 8. PHYSICAL CHARACTERISTICS OF LIQUID ALUMS DELIVERED TO SELECTED WATER TREATMENT PLANTS

Manufacturer/Location of Liquid Alums	Straight pH	Spec. Gravity @ 20°C	Colour/Turb Pt/Co units	Turbidity FTU	% Free Acid	% Free Alumina
1. Border Emo	1.10	1.329	25 clear beige	10	0.25	--
2. Border Kenora New	0.80	1.306	25 clear beige	10	0.85	--
3. Border Kenora Old	0.61	1.326	40 clear beige	20	1.19	--
4. Border Kenora	0.5	1.325	50 clear beige	20	1.23	--
5. Border Rainy River	2.18	1.320	50 clear beige	10	--	0.08
6. Alcan Kingston	2.50	1.325	50 clear beige	20	--	0.28
7. Allied Deseronto	2.25	1.339	210 green/blue, opal.	55	--	0.13
8. Allied Thorold	2.20	1.334	220 green/blue,clear	70	--	0.09
9. Allied Niagara Falls	2.15	1.332	290 green/blue,turbid	70	--	0.07
10. Allied Port Elgin	1.50	1.333	>500 green/blue, very turbid	225	--	0.01
11. Allied Deseronto	2.25	1.328	290 green/blue,turbid	65	--	0.17
12. Allied Hawkesbury	2.15	1.330	140 green/blue, opal	50	--	0.13

TABLE 9. pH OF 1% OF VARIOUS ALUMS AND REDUCTION OF pH AND ALKALINITY CAUSED BY THEIR ADDITION TO DIFFERENT WATERS

Sample	pH of 1% w/v soln.	Alkalinity Reduction per ppm Alum	Initial Reduction of pH	Reduction of pH after 15 min.	Type of Water & Alum Feed
1	3.55	0.45	0.75	0.30	100 ppm alk. 40 ppm alum
2	2.85	0.50	0.85	0.40	100 ppm alk. 40 ppm alum
3	2.7	0.50	1.45	0.90	20 ppm alk. 20 ppm alum

only 0.4 after 15 minutes, even with a low pH alum used at 40 ppm. However, water of a lower buffering capacity (20 ppm alkalinity) showed a substantial loss of 0.9 pH units in 15 minutes, with only 20 ppm of alum. In such waters, the use of a low pH alum could result in a reduction of pH and alkalinity in the water sufficient to impede floc formation.

Because the presence of turbidity can affect the measurement of colour, the turbidity of a sample must be considered when colour is measured. Colour, however does not affect the turbidity measurement when measured on a Hach DR2 spectrophotometer. The variation in colour/turbidity measurement was large, varying from 25 Pt/Co units in samples 1 and 2, to greater than 500 Pt/Co units in sample 10. Alums produced by the direct process were always blue to blue-green in colour and had an opalescence which varied from slight to very heavy, leading to higher colour/turbidity readings than in the beige alums produced by the indirect process. The former alums tended to have higher turbidity readings also, and contained higher levels of the trace metals. The alum, sample 10, with the turbidity of 225 Ftu should not have been accepted for water treatment purposes.

The specific gravity may be the most important physical parameter examined, since it indicates the strength of the liquid alums. Specific gravities varied from 1.306 for sample 2 to 1.339 for sample 11; a specific gravity of 1.335 yields the rated 48.5% w/w concentration that is quoted in the manufacturers specifications. Differences in specific gravity could affect the treatment process. Thus if the same dilution of alum is made, regardless of the specific gravity of the batch the concentration of the solution of the alum could vary greatly. As shown in Table 10 using the regular dilution figure for obtaining a 1% w/v solution, alum of specific gravity 1.306 resulted in a concentration of 9 mg/mL; alum of specific gravity 1.339, when diluted in the same manner, yielded a concentration of 10.15 mg/mL. This difference in concentration could well affect the

TABLE 10. Specific Gravity and Concentration of 1% Solutions of Various Alums

Manufacturer/Location	Specific Gravity	Concentration of 1% w/v solution
Border Chemical (Kenora (New))	1.306	9.0
Allied Chemical (Deseronto)	1.339	10.15
Allied Chemical (Oakville)	1.335	10.02
Allied Chemical (Alexandria)	1.335	10.02

flocculation process.

It is possible that some plant treatment problems have resulted from the use of a new batch of alum with a different pH or specific gravity from that used previously.

Since no methods could be developed to analyse treatment chemicals for asbestos and related fibres, it could not be determined whether chemicals could be a significant source of such material.

Investigations at the water treatment plants revealed that quality deterioration of chemicals can result from improper storage and handling practices. For example, open bin or open bag storage of lime allows moisture and carbon dioxide to produce a build-up of insoluble calcium carbonate; this reduces the potency of the chemical until it may become quite ineffective for its intended purpose.

ORGANIC ANALYSIS
SOLVENT EXTRACTION/GAS CHROMATOGRAPHY

Dinitro toluene was present at low levels in some samples of hydrofluorosilicic acid, the highest level being 2.4 ppm; this compound and other related compounds were recovered from one sample of water plant sludge (R.L. Clark in Toronto), but at levels of less than 0.5 ppm.

Low levels of organic materials were shown to be present by DOC analysis in the primary coagulants, but solvent extraction followed by infra-red spectroscopy was not successful. Results are shown in Table 11. Some treatment aids (unslaked lime, sodium silicofluoride and sodium bicarbonate) showed some unidentifiable organics by the infra-red analysis technique.

Solvent extraction and infra-red analysis on water plant sludges revealed a large number of broad bands in the spectrogram, but no individual organics could be identified. High levels of organics in sludges were

TABLE 11. INFRA RED SCANS AND DOC LEVELS IN ALUM FROM SELECTED WATER TREATMENT PLANTS

Water Plant	DOC (ppm)*	Infra Red
Owen Sound	3.7	NA*
Goderich	4.2	NA
Sarnia	5.0	NA
Elgin Area WS	3.7	NA
R.C. Harris	2.7	NA
Lindsay	2.9	NA
Kenora	1.8	NA
R.L. Clark	2.1	NA
Port Elgin	3.5	NA

*no organics extractable, or sample too corrosive.

also revealed after drying the sludges to constant weight, and then extracting an aliquot with methylene chloride; the methylene chloride extracts were then dried to constant weight and the weight of extracted organics recorded. The weight of extractable organic material is shown in Table 12. The levels of such material ranged from 4 ppm (mg/Kg dried weight of sludge), in the sludge from Kenora, to 11000 ppm at Toronto's Easterly plant. It is interesting that increasing levels of organic extractables were found in the sludges when progressing from Owen Sound to Goderich, Grand Bend and Sarnia southward along the Lake Huron shoreline. A similar trend occurred moving from the Elgin Area System on Lake Erie through to Lake Ontario and Toronto's R.C. Harris and Easterly plants. This corresponds fairly well with increasing urbanization and industrialization of the lakes adjacent to these areas. Kenora would be regarded as non-industrialized.

RECOMMENDATIONS

1. Quality control checks should be carried out on chemicals upon receipt at water treatment plants; if possible, especially for primary coagulants, these checks should include simple tests at the plant, such as pH, colour and colour/turbidity. Manufacturers specifications should conform to AWWA specifications for the chemicals, or such specifications as the Ministry of the Environment may introduce.
2. Unless quality control data and/or in-plant testing indicate an increase in the number of abnormal lots of chemicals of deteriorating quality, monitoring of chemicals need only be undertaken every 5 years.
3. Chemicals must be handled, stored and used correctly at water treatment plants. The Training and Certification Section of the Ministry of the Environment should be requested to provide instruction in the necessary techniques for use and analysis.
4. Results of quality control checks of treatment chemicals must be kept

TABLE 12. INFRA-RED SCANS AND TOTAL EXTRACTABLE ORGANICS IN WATER PLANT SLUDGES

Water Plant	Extractables (ppm)	Infra Red
Owen Sound	10	+*
Goderich	33	+
Grand Bend	360	+
Sarnia	4000	+
Elgin Area W.S.	790	+
R.C. Harris	1780	+
Easterly	11000	+
Kenora	4	+

*organics detectable but not identifiable

at the water treatment plants for a period of at least 5 years; duplicate records should be forwarded to a central file at the Ministry of the Environment.

5. Research into the development of better analytical techniques for the examination of water treatment plant chemicals should be encouraged; this particularly applies to the detection and identification of organic contaminants.

REFERENCES

1. Outlines of Analytical Methods. Ontario Ministry of the Environment, LSB, 1981.
2. Ontario Drinking Water Objectives, MOE - in print.

APPENDIX 1

HANDLING, STORAGE AND ON-SITE TESTING OF CHEMICALS USED IN THE TREATMENT OF DRINKING WATER

The information given in this Appendix is intended as a guide for the water plant operator or supervisor. Included with details regarding the recommended handling and storage procedures, are recommendations for testing procedures to be carried out on chemicals upon receipt at the water plant, along with the appropriate AWWA Standard number. When ordering treatment chemicals, a request should be made that they conform to AWWA standards. Manufacturers should provide results of the testing for conformity with the specifications. Water plants should occasionally submit samples of chemicals received, for spot checking of specifications, to the Ministry of the Environment laboratories. Chemicals used in water treatment should contain no substances in quantities capable of causing injurious effects upon the health of those consuming the water to which the chemical has been added.

1. PRIMARY COAGULANTS

A) ALUMINUM SULPHATE ($Al_2(SO_4)_3$), AWWA Standard B403-70.

Aluminum sulphate (liquid or solid) is used for coagulation.

1. LIQUID

Storage - Liquid alum should be stored in lined steel, stainless steel, lead, rubber or plastic containers. Liquid alum should be shipped and stored at temperatures above -13.3°C; below this temperature the alum will crystallize out. Although it appears to redissolve, if warmed, the alum will never subsequently remain permanently in solution and its suitability for treatment will be compromised.

Precautions - Liquid alum is of low pH and corrosive; it is irritating to the skin; handling requires caution and the use of protective clothing and corrosion-resistant equipment.

Testing - The following visual and analytical tests should be carried out on liquid alums upon delivery at the plant.

a) Colour - alum should be beige or blue/green in colour; Alums which are bluish or bluish-green in colour are probably manufactured by the direct process; this process tends to produce alums with a higher trace metal content. Colour should be measured with a Hach DR2 spectrophotometer. The reading for colour using this instrument, is actually colour/turbidity since the latter interferes with the measurement of the former.

b) Turbidity - liquid alum should be sufficiently clear to enable flow measuring devices to be read without difficulty. Alums with turbidity, measured on a Hach DR2 spectrophotometer, exceeding 200 Ftu should be regarded as unsatisfactory.

c) pH and pH of 1% solution - excessively low pH in liquid alum could cause treatment problems, especially in low alkalinity waters; the pH of a 1% solution of it, should thus be measured to determine if it is significantly different from previous shipments.

Specifications. Manufacturers specifications should include results of the following tests; the AWWA specifications are given in parenthesis:

Available water soluble aluminum as Al (not less than 4.25%)

Available water soluble aluminum as Al_2O_3 (not less than 8%)

Excess water soluble Al_2O_3 (at least 0.025%)

Excess water soluble aluminum (at least 0.013%)

Total water soluble iron as Fe_2O_3 (no more than 0.35% based on 8% Al_2O_3).

Insoluble and Suspended Material (not to exceed 0.2%)

2. SOLID

Storage - Solid alum should be stored in a dry area, protected from moisture in sealed containers

Precautions - Handling of solid alum requires caution and the use of dust masks

Testing:

a) The size of solid alum is important whether it is to be dissolved or fed with a dry feeding machine. Sizing may be done with sieves, in which case 100% should pass through a 3" ring and 75% be retained on a 1/2" sieve (US standard Z23.1 - 1961).

Specifications. Manufacturers specifications should include results of the following tests; the AWWA specifications are given in parenthesis:

Available water soluble aluminum as Al (not less than 9%)

Available water soluble aluminum as Al_2O_3 (not less than 17%)

Excess water soluble Al_2O_3 (at least 0.05%)

Excess water soluble aluminum (at least 0.026%)

Total water soluble iron as Fe_2O_3 (no more than 0.75% based on 17% Al_2O_3)

Insoluble and suspended material (not to exceed 0.5%)

B) LIQUID FERRIC CHLORIDE ($FeCl_3$)

There are no AWWA Standards for ferric chloride; it is used as a coagulant.

Storage - Liquid ferric chloride should be stored in lined steel, stainless steel or plastic or rubber containers; it is normally supplied as a 35-45% solution.

Precautions - Liquid ferric chloride is stain-producing, corrosive and irritating to eyes, lungs and skin. Protective clothing and corrosion-proof equipment is necessary during handling.

Testing -

a) Ferric chloride from new suppliers should be analysed for heavy metals; water plants should send samples to MOE Central Laboratories for analysis.

b) Turbidity - liquid ferric chloride should be sufficiently clear to enable flow-measuring devices to be read without difficulty.

Specifications. Lquid ferric chloride should contain from 32-47% $FeCl_3$ and 11-17% iron.

C) SODIUM ALUMINATE ($Na_2O \cdot Al_2O_3$)

Sodium aluminate is used for coagulation and for corrosion and pH control. There are no AWWA standards for this treatment chemical.

Storage - The solid form may be stored in bags, the liquid in steel or plastic drums.

Precautions - The dust is harmful and protective masks should be used during handling. The chemical tends to cake, and is mildly corrosive when wet.

Testing -

a) Turbidity - solutions of sodium aluminate should be sufficiently clear to enable flow-measuring devices to be read without difficulty.

Specifications. Solid sodium aluminate should contain 55% Al_2O_3, and liquid 32% Al_2O_3.

2. TREATMENT AIDS

Contaminant concentrations in chemicals used as treatment aids are low, and not expected to contribute levels of concern to the treated

water, especially in view of the low dosage and, for the most part, intermittent use. Testing of such chemicals upon receipt at water treatment plants is not necessary. However, storage and handling procedures are given below, along with the AWWA specifications and standard number for each chemical.

A) SODIUM CARBONATE (Na_2CO_3) SODA ASH AWWA STANDARD B201-80

Sodium carbonate is used for corrosion control, softening and as a coagulant aid.

Storage - Sodium carbonate in bags or drums, should be stored in a cool, dry area in sealed containers to minimize contact with moisture and air; if moisture is absorbed, the material will become lumpy and difficult to handle and dissolve.

Precautions - Not classified as dangerous but it is dusty and irritates the eyes, nose, lungs and skin; it is mildly corrosive if wet. Goggles and dust respirators are recommended for handling. Soda ash and lime should not be present together, since caustic soda (highly corrosive) may form on combination of the chemicals and moisture.

Specifications. Material should be free of dirt, rocks, twigs or other foreign materials. Manufacturers specifications should provide results of the following tests; the AWWA specifications are given in parenthesis.

Density - light soda ash (0.5 - 0.8 g/mL)

- dense soda ash (0.9 to 1.1 gm/mL)

Sodium carbonate (not less than 99% by weight Na_2CO_3)

Sodium oxide (equivalent of not less than 57.9% Na_2O)

Insoluble material (not to exceed 0.05%)

B) SODIUM BICARBONATE $NaHCO_3$

There are no AWWA Standards for sodium bicarbonate. It is used as an aid in coagulation and for corrosion control.

Storage - Sodium bicarbonate should be protected from moisture in plastic-lined bags and stored in a cool dry place.

Precautions - It should not be allowed to come into contact with aluminum, with which it reacts, otherwise no special handling precautions are necessary.

Specifications. Sodium bicarbonate as $NaHCO_3$ not less than 98.6%.

C) QUICKLIME AND HYDRATED LIME CaO AWWA STANDARD B202-77

Lime is used for pH and corrosion control and for softening.

Storage - Lime should be stored in a cool dry area in sealed containers to minimize contact with moisture and air; it is unstable if left out in the open in unsealed containers.

Precautions - Lime is not classified as a dangerous chemical, but goggles and dust respirators are recommended for handling. Lime and soda ash should not be present together, for caustic soda may form if the chemicals combine in the presence of moisture. The chemical reacts with water when it is mildly corrosive and can burn the skin.

Specifications. Lime is supplied as lumps (density 897-1040 kg/m^3), pebbles (density 960-1024 kg/m^3) or granules (density 800-1120 kg/m^3). Manufacturers specifications should include the results of the following tests; the AWWA specifications are given in parenthesis:

Available calcium oxide (quicklime not less than 80%, hydrated lime not less than 62%)

Insoluble material (not more than 5%)

Slaking test (quicklime shall produce at least a 10^oC rise in

temperature in 3 minutes, and a maximum temperature within 20 minutes).

Fluoride (such that the fluoride content in distributed water will not be increased more than 0.1 mg/L on lime treatment)

D) ACTIVATED CARBON

Activated carbon is used for taste and odour control organics removal and for dechlorination.

1. POWDERED ACTIVATED CARBON. AWWA STANDARD B600-78

Storage - Powdered activated carbon should be stored in a fireproof building, stacked in rows with aisles between so that each individual bag is accessible for removal in case of fire. No other materials should be in the same building.

Precautions - Strict precautions to avoid contact with strong oxidizing agents (chlorine, hypochlorites, permanganate, ozone and peroxides) are necessary, and mixing with hydrocarbons may cause spontaneous combustion. Dust respirators should be used in handling, and electrical connections in feed rooms should be water-tight and dustproof. When moist, activated carbon will absorb oxygen from the air, and appropriate safety measures should be observed when entering enclosed areas containing activated carbon. N.B. A carbon fire may be extinguished using a fine water spray or chemical foam; water jets will spread the smouldering particles.

Specifications. The material should be free of diluents, either soluble or insoluble, and should not impart to the water any contaminants that exceed the limits of the Drinking Water Objectives when combined with levels already in the water. Manufacturers specifications should include the results of the following tests; the AWWA standard is given in parenthesis:

Moisture content (not to exceed 8% by weight at time of shipment)

Apparent density (not less than 0.2 gm/mL nor greater than 0.75 gm/mL)

Absorptive capacity

a) Iodine number (not less than 500)

b) Phenol value (not greater than 3.5)

N.B. Reduction of threshold odour or tannin absorption can be used to measure absorptive capacity. (Reduction of threshold odour by a sample, should be not less than 70% of that of a reference sample, and the tannin value not more than 10% greater).

2. GRANULAR ACTIVATED CARBON AWWA STANDARD B604-74

Storage - Granular activated carbon should be stored in a cool dry area.

Precautions - Strict precautions should be taken to avoid contact with strong oxidizing agents (chlorine, hypochlorites, permanganate, ozone and peroxides, mixing with hydrocarbon may cause spontaneous combustion. When moist, an oxygen-depletion hazard may develop in confined areas.

Specifications. The material shall contain no soluble inorganic or organic substances that would render the treated water unfit for use, and should not impart to the water any contaminants that exceed the limits of the Drinking Water Objectives when combined with levels already in the water.

Manufacturers specifications should include the results of the following tests; the AWWA standard is given in parenthesis:

Moisture content (not to exceed 8% by weight at time of shipment)

Apparent density (not less than 0.36 gm/mL)

Abrasion resistance (retention of average particle size not less than 70% as determined by standard tests)

Absorptive capacity

a) Iodine number (not less than 500)

E) SODIUM HYDROXIDE (NaOH) AWWA STANDARD B501-80

Sodium hydroxide is used for pH and corrosion control.

1. ANHYDROUS SODIUM HYDROXIDE (CAUSTIC SODA)

Storage - Flake, ground, crystal or bead caustic soda should be stored in steel containers, fibreboard drums or polyethylene-lined bags, in a cool, dry area. Protection from moisture is essential, since it will be absorbed, generating heat.

Precautions - Caustic soda in all forms is extremely hazardous and full protective clothing including goggles, face shields, cotton clothing, rubber aprons, boots and gloves are essential. Dilution and dissolution result in heat-generation, and should be carried out with extreme caution to avoid boiling or splattering; emergency eyewash and shower facilities should be provided in the vicinity of handling areas. All personnel should be given detailed instructions on handling procedures.

Specifications. Caustic soda is a white to off-white opaque to translucent solid which rapidly absorbs moisture. Manufacturers specifications should include the results of the following tests; the AWWA standard is given in parenthesis:

Percent total alkalinity as Na_2O (74.4% minimum)

Percent sodium hydroxide as NaOH (96% minimum)

Percent carbonate as Na_2CO_3 (not more than 2%)

2. LIQUID SODIUM HYDROXIDE (CAUSTIC SODA)

Storage - Liquid caustic soda should be stored in glass or steel containers in a cool dry area.

Precautions - Caustic soda in all forms is extremely hazardous and full protective clothing, including goggles, face shields, cotton clothing, rubber aprons, boots and gloves are essential. Dilution results in heat-generation; and should be carried out with extreme caution, to avoid boiling or splattering; emergency eyewash and shower facilities should be provided in the vicinity of handling areas. All personnel should be given detailed instructions on handling procedures. The 50% NaOH begins to crystallize at $12^{o}C$, which can cause problems in Canadian winters.

Specifications. Caustic soda is available in liquid form in concentrations from 12% to 50% NaOH. Manufacturers specifications should include the results of the following tests; the AWWA standard is given in parenthesis:

Percent total NaOH (at least 50%)

N.B. In Canada, percent total NaOH should be as specified in the order.

F) FLUORIDATION CHEMICALS

Fluoridation chemicals are added for prophylactic purposes to prevent dental caries in consumers.

1. HYDROFLUOROSILICIC ACID (H_2SiF_6) AWWA STANDARD B703-71

Storage - Hydrofluorosilicic acid (fluosilicic acid) should be stored in a well-ventilated room in containers of structural carbon, Hastolley C, Durimet 20 or such rubbers and plastics as recommended by the manufacturers.

Precautions - Since hydrofluorosilicic acid is very corrosive and harmful to the skin and eyes, protective clothing and eyewear are essential. Feed equipment should be the same as that specified for storage containers. Emergency showers should be available in the vicinity of handling areas.

Specifications. Hydrofluorosilicic acid should be clean and free of visible suspended matter, and generally contains from 20 to 30% H_2SiF_6. Manufacturers specifications should include the results of the following tests; the AWWA specifications are given in parenthesis:

Heavy metals (mercury, lead, bismuth and copper expressed as lead) (not more than 0.020%)

Percent H_2SiF_6 by weight (between 20 and 30%).

2. SODIUM SILICOFLUORIDE (Na_2SiF_6) AWWA STANDARD B702-74

Storage - Fibre drums, paper bags or barrels may be used to store sodium silicofluoride in a cool dry place; it is hygroscopic and tends to lump, rendering use difficult.

Precautions - Sodium silicofluoride is poisonous if inhaled or swallowed and containers should be labelled appropriately. Dust respirators and goggles should be worn during handling and personnel should wash thoroughly after handling the material. Spills should be cleaned up immediately. Medical attention is required immediately if the solid chemical is ingested.

Specifications. Sodium silicofluoride is a white powder, and should contain no stones, sticks, paper or other foreign matter. It may contain an anti-caking agent if this will not interfere with its application to drinking water, and it is non-toxic and odourless. Manufacturers specifications should include the results of the following tests; the AWWA specifications are given in parenthesis:

Density (approximately 75 lb/cu. ft.)

Standard sieve #40 (420 μm) (at least 98% passing through)

Standard sieve #325 (44 μm) (less than 25% passing through)

Sodium silicofluoride (Na_2SiF_8) (minimum 98% corresponding to approximately 59.4% fluoride ions)

Insoluble matter (not to exceed 0.5%)

Moisture (not to exceed 0.5%)

Heavy metals (mercury, lead, bismuth and copper expressed as lead) (not more than 0.05%).

NOTE. Much of the material used to produce this Appendix was obtained from the AWWA Standards for the individual chemicals (American Water Works Association, 6666, West Quincy Avenue, Denver, Colorado, 80235).

These standards include such information as:

-Definition of the Standard and an outline of the basis for the purchase, and/or rejection of the chemical

-Description of the material, including impurities and size

-Sampling, packing, shipping and marking requirements for various chemicals

-Testing methods to determine the quality of chemicals

A Wall Chart, listing common water treatment chemicals, chemical names and formulae, containers precautions uses etc. in abbreviated form is available from "Water and Pollution Control", 1450, Don Mills Road, Don Mills, Ontario, M3B 2X7 at $5.00 each (bulk rates available).

Experimental and Environmental Modelling Studies of Hazardous Substances in Ontario

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ABSTRACT

It is becoming widely accepted that assessment of the behaviour, and hence the effects, of toxic chemicals requires the use of mathematical models which may be applied to actual or evaluative environments. This is necessitated by the large number of potential substances of concern and the wide variation in their physical-chemical and toxicological properties. In this project a version of the evaluative Fugacity Model has been developed and is being applied to a number of selected chemicals, namely the chlorobenzenes, the chlorophenols, mirex, atrazine and trichloroethylene. The evaluative model is specifically designed to be applicable to the Ontario environment. Attempts are being made to obtain emission rate data and environmental monitoring data which can be used to calibrate the model.

A novel aspect is quantification of concentration heterogeneities and the inclusion of such concentrations as lognormal or Weibull distribution functions. The feasibility of presenting the data in computer - generated colour contour form is being investigated.

Finally, methods of including toxicity information are being examined and will be discussed.

INTRODUCTION.

In late 1982 we initiated a project with MOE support to develop a computer modelling system which could be used to assess the behaviour, and ultimately effects, of toxic contaminants in Ontario. This is essentially a progress report and a description of current and future work.

It is now clear that environmental "management" of toxic substances presents a severe scientific and organizational problems to Government Agencies. It is an issue quite unlike any previous issue which has been successfully addressed. Some features which cause difficulties are:-

1. There is a large number of recognized and potential toxicants, most lists running to several hundred members.
2. In many cases the "member" is a homologous series, for example the 209 polychlorinated biphenyls, or the chlorinated dibenzofurons, dibenzodioxins or toxaphenes.
3. Rarely is there adequate information on the partitioning, reaction and inter-media transport properties of these substances.
4. Rarely is there adequate toxicological information, nor is there an appreciation of the dominant routes of exposure or dosage.
5. Many of the contaminants migrate readily between air, water, soil, sediment and biota thus they do not fit neatly into organizational structures designed to assess "water or air pollutants" such as phosphorous or NO_x.
6. The environment is a very complex assembly of media of

varying composition and condition (e.g. temperature).

7. Sources are usually of unknown magnitude may be "point" or "non-point" or both in nature and nay be intermittent in nature. Certain locations may be particularly contaminated.
8. Monitoring data are sparse, expensive to obtain and may stretch "state of the art" analytical methods.

It is clear that the resources which can be devoted to any one toxicant must be relatively modest and can not approach those which have been devoted to toxicants such as mercury, phosphorus, or sulfur oxides.

We have become convinced that the only realistic approach is to develop a number of mathematical models or partial representations of reality in which the general behaviour of each toxicant can be computed from known input parameters. The art of such modelling is to devise models in which the correct key processes are described and less important processes are ignored or appropriate simplifying assumptions made. The critical decision is "what to leave in" vs "what to leave out". This requires, judgement based on experience.

In a series of recent publications (Mackay and co-workers 1978-83) we have advocated the use of the fugacity concept for simplifying environmental modelling. Accordingly, in this project we are extending these models and applying them to Southern Ontario conditions for selected substances.

The general aim is to gather information on the following aspects for a defined study area and synthesise them into a model which can be subjected to some form of validation

(i) Source locations, amounts, and emission times, including advective inflow in air and water (especially the Niagara River).

(ii) Relevant physical chemical properties.

(iii) Toxicological properties.

(iv) Monitoring data in the study area.

We discuss below each aspect in turn.

Study Area/Evaluative Model

Two models have been assembled. First is a purely evaluative model comprising the various environmental compartments described by Neely & Mackay (1982) as shown in Figure 1. This is the closest available to a standardised evaluative environment. Second is a model representing part of Southern Ontario as shown in Figure 2. The boundaries are somewhat arbitrary but it is believed that they include most of the regions of substantial emission and concern. The French River forms the northern boundary. All of Lake Ontario and Lake St. Clair are included as well as Lake Erie north of the international boundary, Georgian Bay and part of Lake Huron. This region may be redefined later if it is considered necessary. Lake Ontario is the only one of the Great Lake included along with Lake Simcoe and the other inland lakes. In total the area is approximately 13 x 10^{10} m^2 of land and 5.0 x 10^{10} m^2 of water containing these estimated volumes (assuming reasonable depths, heights and volume fractions).

air	3.7×10^{14} m^3
water	5.0×10^{12} m^3
soil	2.0×10^{10} m^3
sediment	1.5×10^{9} m^3
suspended sediment	2.5×10^{7} m^3
aquatic biota	5.0×10^{6} m^3

The Benchmark Chemicals

Following discussions with the Ministry we have selected the following chemicals for study. In each case the chemical(s) have a dominant environmental fate property as indicated

mirex	sediment contaminant
atrazine	herbicide, water and soil contaminant
trichloroethylene	air contaminant
chlorobenzenes	series of contaminants of varying hydrophobicity tending to accumulate in air or sediments and aquatic biota.
chlorophenols	series of water contaminants of varying hydrophobicity of unusual toxicity, precursors to dioxins

It is possible that other substances may be added at a later stage if appropriate.

Physical-Chemical Properties

An extensive literature search has been conducted for these compounds and all available physical-chemical property data assembled. In some cases it has been necessary to measure the properties.

The reports obtained from this literature review have been standarized to take the following format.

A general narrative is written describing the properties and other features of the chemical, including review (with references) of other studies, and matters of historical and other significance. At various stages the narrative is punctuated by "data sheets" which give the following information in a format which may be directly incorporated into the fugacity models.

(1) Sources (emissions categorized into air, water and soil in mol/h for the Province and the evaluative environments).

(2) Fugacity Capacities (Z values) (partitioning information between phases).

(3) Reactivity (k values) (first order degradation rate constant for each relevant reaction in each phase).

(4) Advection Information on "background" concentrations and flows in air and water.

(5) Interphase transport data (D values), Mass transfer coefficients and/or uptake times.

(6) Hazard/Toxicity Information on concentrations and durations or uptake rates in air, water and food which have been assessed as having defined (or no) adverse effects.

(7) Monitoring Data Information on prevailing concentrations in the Province in various compartments in histogram form of frequency versus concentration.

Reports have been written in draft form for all substances.

When compiling data on the properties of the substances, we have found that there are considerable variations in the values reported, especially for solubility, vapour pressure, octanol-water, sorption and bioconcentration partition coefficients. We have devised a system for compiling the data, weighting it according to perceived reliability then selecting the best set of mutually consistent values. This has proved to be an interesting aspect and will, we believe, be of value to the Ministry (and others) in critically assessing physical chemical property data which may be received or measured for compounds of environmental interest.

Essentially, the method is an application of the maximum likelihood principle to the data. We have sought assistance from Professor Alan Wilks of the Department of Statistics at Princeton University in this regard.

Fugacity Models

A modified level III fugacity model is being compiled which is appropriate for application to Southern Ontario. The compartments included are: - soil, water (including Lake Ontario), air (to a height of 2 km), sediments, suspended sediment, atmospheric particulates, aquatic biota and terrestrial biota. The simplest level I and II models are also being compiled. Degradation reactions in all phases can be included. Interphase transport reactions included are the exchanges between: - air and water (by diffusion and rainfall), air and soil (by diffusion and rainfall), air and atmospheric particles, air and terrestrial biota, soil and water (runoff), soil and terrestrial biota, water and suspended sediment, water and aquatic biota and sediment and water, suspended sediment and (bottom) sediment atmospheric particles and water, atmospheric particles and soil. Provision is made for the introduction of chemical into air, water and soil compartments. Background or "out of Province" inputs may also be included.

This model is being programmed in BASIC on our IBM Personal Computer and should be readily transferable to other systems.

It is also planned to develop a Level IV model which can be used to obtain data on the time-response characteristics of each phase.

Treatment of phase heterogeneities and incorporation of monitoring data.

A difficulty which arises from the fugacity model is that the phase concentrations obtained are homogeneous, i.e. a constant value applies at all locations. In practice, of course, there are wide variations in concentrations depending on the proximity to sources. This is reflected in the monitoring data which show this range of concentration.

Our approach to treating this "problem" is to compute separately a distribution of concentrations in each principal compartment of air, soil, sediment and water. The other compartments, while important as transfer media (e.g. atmospheric particles) or for socioeconomic reasons (e.g. fish) are not significant "sinks" for chemicals in a mass sense. Their concentrations generally reflect the concentrations in the dominant surrounding compartment (e.g. fish in water). We have investigated the use of normal, log-normal and Weibull distribution functions for characterizing concentration variations within a phase. A constraint must be satisfied that the total amount in a compartment is that indicated by the model (i.e. the same mean concentration applies) but the "variance" (or equivalent) can be selected as appropriate in each case.

We have found that the Weibull distribution is particularly convenient because it exists in analytical form in both differential and integral versions. An analysis has been undertaken of mirex concentration data in the sediments of Lake Ontario. A further stage is the assignment of the concentration distributions to specific locations within the Lake using knowledge of source locations and current directions. This enables a concentration contour diagram to be devised (using available computer graphic techniques) and compared with the actual data obtained by Thomas et al (1983). Figures 3 and 4 illustrate this approach. We hope to extend it to other phases such as air and water.

Hazard Assessment

This is the most distant goal and at present we have only a rather indistrinct impression of the system which may be advised. It is appropriate, however, at this time to expose present ideas for criticism and comment.

From the fugacity models come assessments of the likely prevailing chemical concentrations in air, water, fish, etc., which arise from specified emissions. This could take the form of statements of the form: - The average concentration of compound x will be y mol /m^3 in water but with a distribution of concentrations as shown in Figure 5. Figure 6 illustrates the distribution of mirex concentration in the sediment of Lake Ontario fitted to a Weibull curve. (Thomas 1983).

From a review of the compound's toxicology comes an adverse effect distribution of concentrations ranging from an acute toxicity at the upper end to a "no effect level" or (lower) guideline or regulatien for "acceptable" water concentrations at the lower end. Juxtaposing these two concentration distributions as is shown in Figure 7 reveals the proximity of the prevailing (or potential) Provincial concentration distribution to the range of concentrations of toxicoloical concern. If the two ranges are close (or overlap) it is apparent that some parts of the Province are (or may be) exposed to undesirably high exposures through the water environment. As the ranges more apart the solution becomes less serious. The seriousness of the situation is thus expressed as the proximity of these concentration distributions. No attempt is made to predict effects e.g. incidence of disease, since this is probably impossible. It should be possible to establish relative priorities e.g. if the distributions for benzene are closer than for naphthalene, benzene is probably a more problematic chemical and may deserve more attention.

This analysis can be applied separately to the air, water and food (i.e. terrestrial and aquatic biota) compartments. An assessment can be made of uptake or exposure to each medium (e.g. a) g/yr by air breathing, b) g/yr by water drinking, c) g/yr by fish eating, d) g/yr by meat eating, etc. The dominant exposure medium then becomes apparent. It is suspected, for example, that this may be air for trichloroethylene, water for chlorophenol,

and fish for hexachlorobenzene. More attention can then be focussed on that medium and on areas of the Province where that medium is found to be, or suspected to be most contaminated. It may then be appropriate to devote more monitoring effort to these regions and compartments.

In most cases the conclusions will be self-evident and it is suspected that existing monitoring programs already take these factors into account on a "common sense" basis. For some chemicals data gaps may be revealed and for new chemicals a prior assessment can be made of likely dominant routes of human exposure. Situations which have been accidentally overlooked may be identified.

It is emphasised that at this point we are far from having achieved this goal but we believe that it is useful at this stage to expose these ambitions for critical comment.

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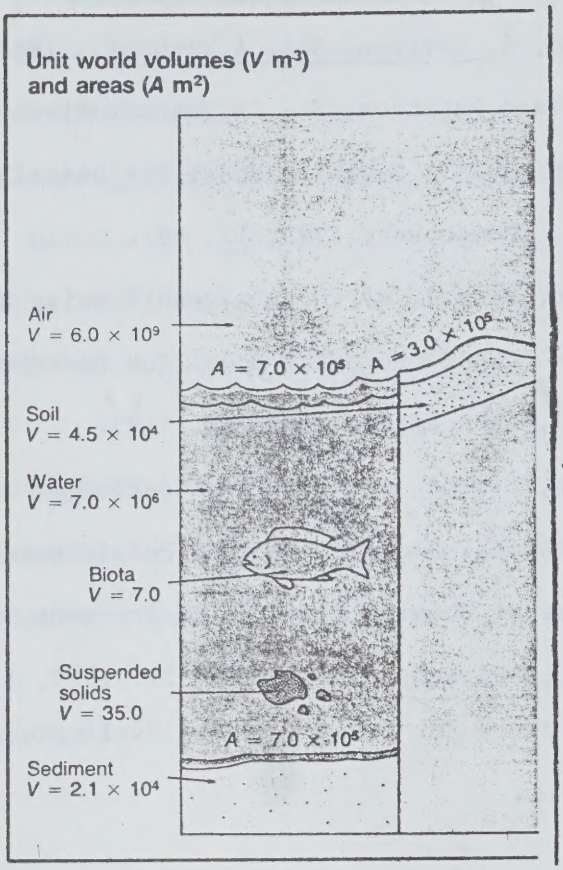


Figure 1. Evaluative Model or Unit World

Figure 2. Map of Southern Ontario Study Area

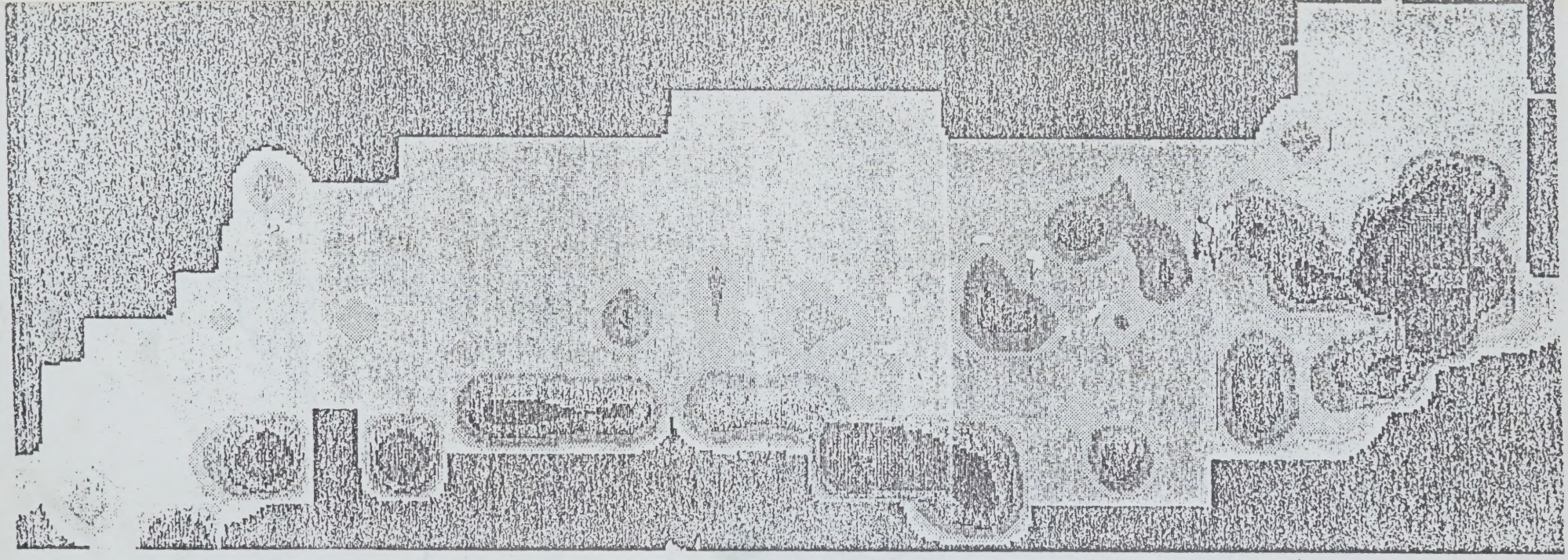
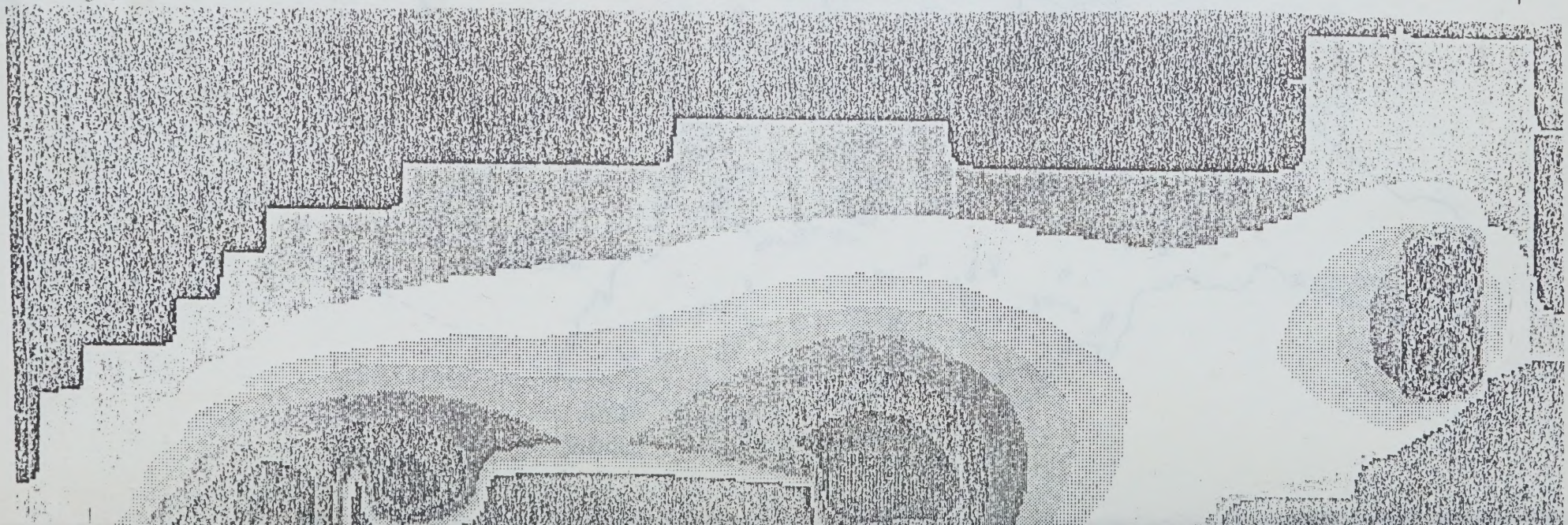


Figure 3. Actual Concentrations of mirex in sediment of Lake Ontario (Thomas 1983)

Figure 4. Predicted concentration of mirex in sediment of Lake Ontario



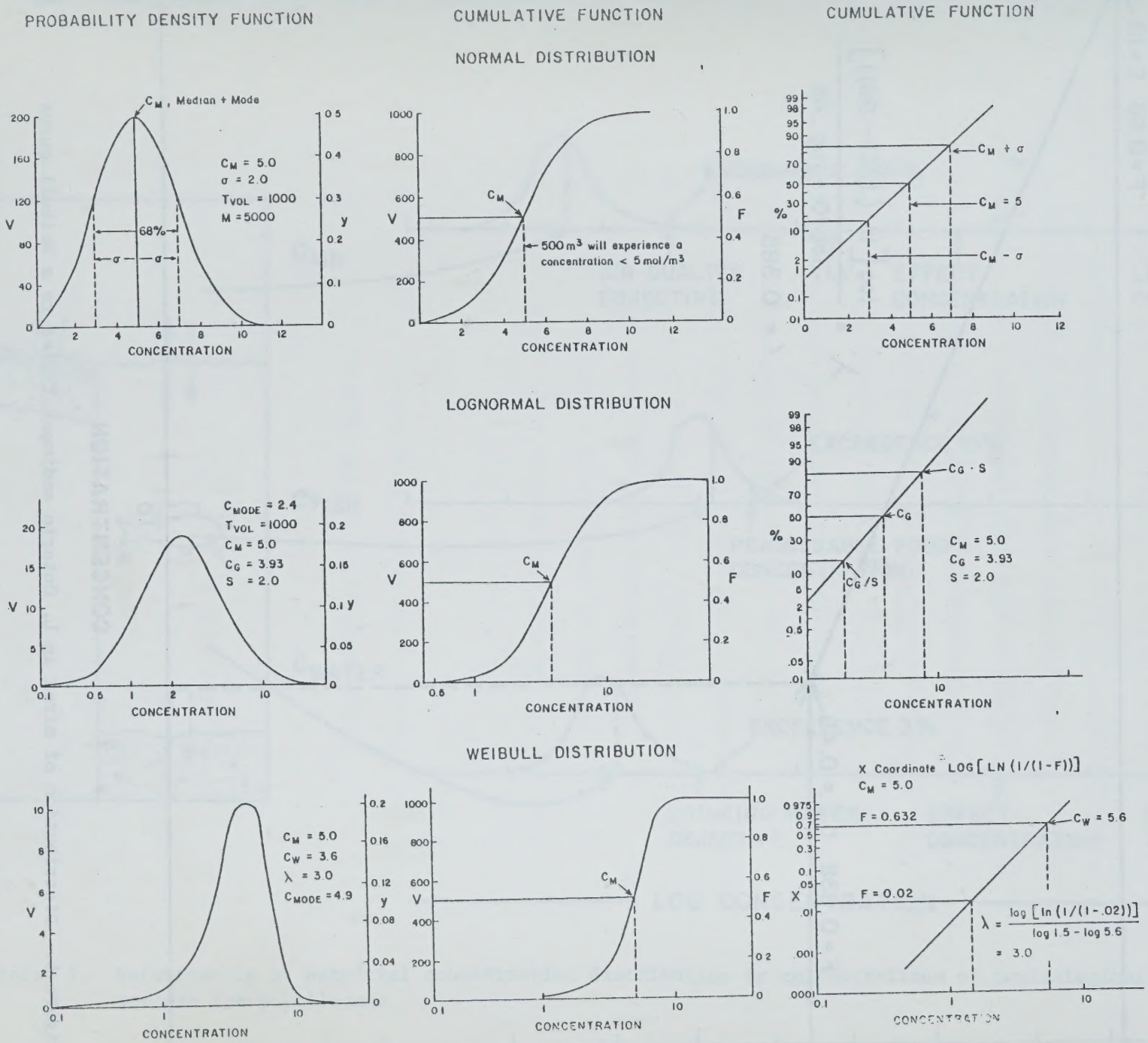


Figure 5. Distribution Functions for characterising concentration variations within a phase

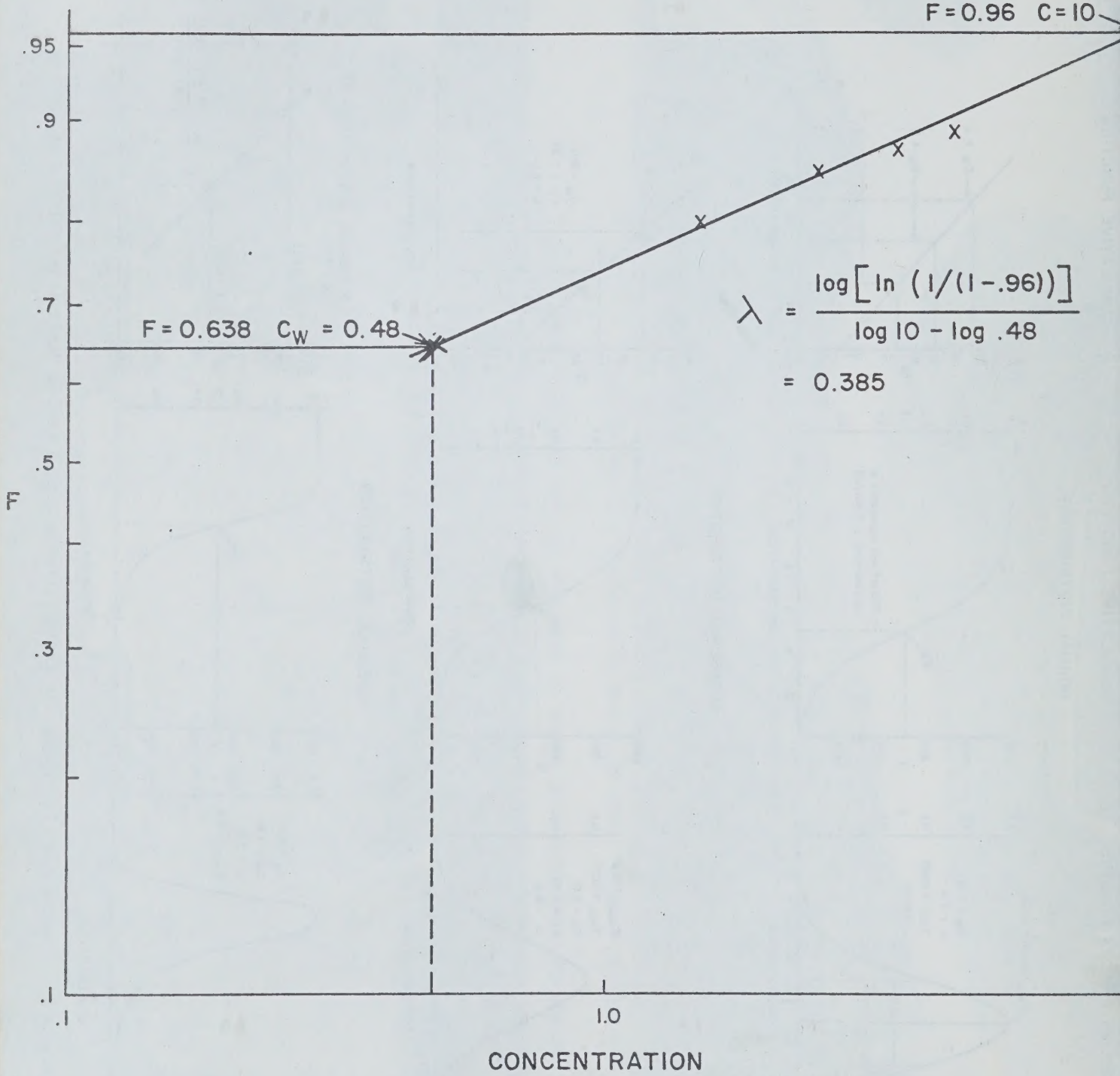


Figure 6. Distribution of mirex in L. Ontario sediment fitted to a Weibull curve

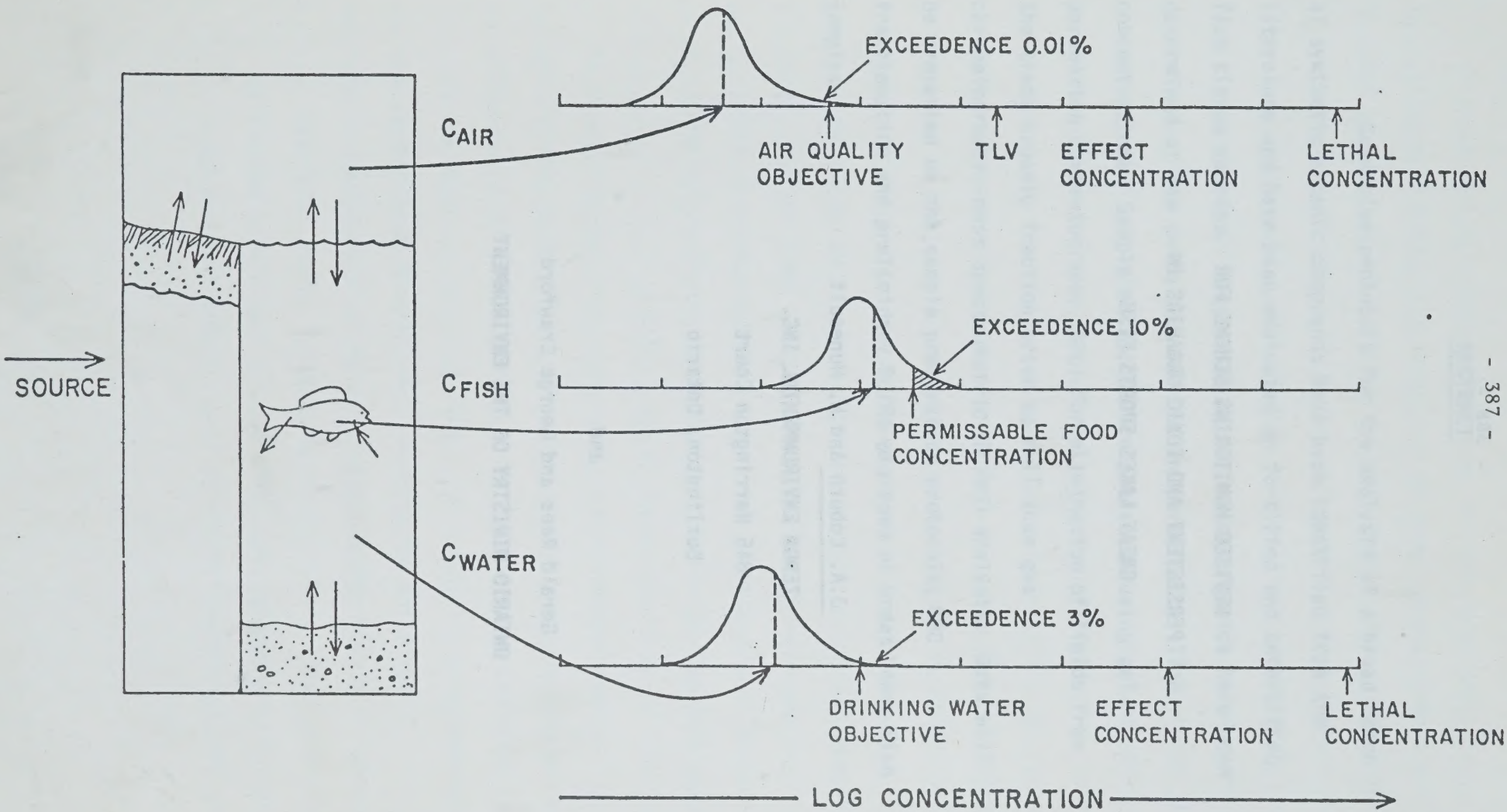


Figure 7. Relationship of potential concentration distribution to concentrations of toxicological concern for a pollutant

REVISED MONITORING SCHEME FOR PERSISTENT AND TOXIC ORGANICS IN GREAT LAKES SPORTS FISH

by

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ONTARIO MINISTRY OF THE ENVIRONMENT

ABSTRACT

Screening protocols for the analysis of a broad range of synthetic organic compounds have been identified from the literature and have been evaluated on fortified and unfortified fish tissue samples. Recoveries of the trace organics have been determined at low parts per million and parts per billion concentrations. Sample extracts were processed using gel permeation chromatography (GPC) for elimination of lipids from the trace organic fraction prior to full scan gas chromatographic-mass spectrometric (GC/MS) analysis. Data will be presented on the sample preparation protocols, GPC fractionation and preliminary GC/MS analyses of Great Lakes fish samples.

Over the past two decades the number of detections of trace organic contaminants in fish samples has been increasing considerably. These analysis have detected the presence of PCB, DDT and metabolites, chlordane, benzene hexachloride isomers including lindane, dieldin, endrin, chlorinated benzenes, mirex, mirex metabolites, and chlorinated styrenes (COA Report, 1981). The International Joint Commission has reported that in addition to the usual chlorinated hydrocarbons, phthalic acid esters, volatiles such as chloroform, bromoform and tetrachloroethylene, Toxaphene, polyaromatic hydrocarbons (PAH) and alkylated and chlorinated PAH and a broad array of aliphatic hydrocarbons have been identified in fish.

Numerous authors have more recently reported the presence of 2,3,7,8-tetrachlordibenzo-p-dioxin at part pere trillion concentrations (Kuehl, 1981; Ryan, 1982; Harless, 1980). Additional analysis have also revealed the presence of ultra-trace (part per trillion) levels of other chlorinated dioxins and polychlorinated-dibenzofurans (Kuehl, 1981).

Kuehl,(1980) has also reported the presence of hexachloro and heptachloro styrenes, pentachlorophenol and pentachlorobenzyl alcohol in fish samples in the Great Lakes watershed.

Chlorinated toluenes and chlorofluorotoluenes have been reported in fish tissue samples collected from the Niagara River (Yurawecz, 1979). Polychlorinated diphenyl ethers have been reported in sediments and fish collected from Whitby Harbour in Lake Ontario (Coburn 1981) with levels higher than the PCB.

Polychlorinated naphthalenes have been detected and identified in fish from the Titabawassee River which flows into Saginaw Bay of Lake Huron (Kuehl, 1980) at very low parts per billion concentrations.

Hexachlorobutadiene and hexachlorocyclopentadiene have been reported to be in numerous hazardous waste landfills along the Niagara River in the New York State (Intereagency Task Force on Hazardous Wastes, 1979) however only the hexachlorobutadiene was detected in the fish samples from the Niagara River (Yurawecz, 1979).

A comprehensive analysis of water and sediments adjacent to hazardous waste landfills in Niagara Falls, N.Y. (Elder, 1981) has detected chlorobenzenes, chlorotoluenes, polyaromatic hydrocarbons, PCB, chlorophenols, fluorinated aromatics, and a series of unchlorinated and chlorinated benzyl derivatives. There have been no reports in the literature of the detection of this latter group of organics in fish samples from Niagara River or Lake Ontario.

The report of Hesselberg, (1982) also identified two halogenated contaminants found in Great Lakes fish samples which were not previously identified as pollutants of concern by the International Joint Commission. They were 3-chloro-1-propynyl-cyclohexane and 3-bromomethyl-cyclohexene with the latter compound being detected in all Great Lakes samples tested.

These reports reveal that as analytical techniques continue to improve, there are increasing detections of trace organic chemicals in the fish of the Great Lakes at ever decreasing concentrations.

There have been basically four main techniques employed in the extraction of persistent and toxic organics such as DDT, chlordane, PCB, mirex, etc. from biological tissues. These are:

i) mechanical homogenization of the tissue with a solvent or mixture of solvents,

ii) cold HCI acid dissolution of the biological tissue followed by a partitioning into an organic solvent.

iii) solvent elution from a column of biological tissue mixed with anhydrous sodium sulphate,

iv) steam distillation of biological tissue (in an aqueous solution) followed by condensation of the water vapour and trace organics and flow through partitioning of the organics into a non-water soluble solvent.

The first three techniques, because of the presence of high levels of lipids and fats, require the separation of these coextractives from the extract prior to separation or fractionation of contaminants and anlysis. The two most commonly employed techniques for lipid - contaminants separation are:

i) acetonitrile - petroleum ether partitioning usually followed by Forisil column chromatography (This technique is referred to as the Mills, Onley, Gaither technique).

ii) automated gel permeation chromatography on Bio-Beads SX-3 eluting with dichloromethane or dichloromethane; cyclohexane solvent mixtures.

EXPERIMENTAL SECTION

Fortified "clean" fish and unfortified "real" fish samples were analyzed using three analytical techniques. Model compounds, representing five chemical classes were used for fortification at concentration levels of roughly 100 ng/g and 20 ug/g. The "clean" sample was a lake trout from Lake Opeongo in Algonquin Park and the "real" sample was a lake trout from the north shore of Lake Ontario. Table 1 shows the model compounds and surrogate standards used in this study while Table 2 shows the concentrations of these compounds at the two fortification levels.

For the analysis of the high level spiked samples 5 grams of tissue were extracted and the extract taken to a final volume of 1.0 mL. For the low level spike and the unspiked "real" fish 5-10 grams of tissue were extracted and the final extract volume adjusted to 200 uL.

The three techniques evaluated are briefly presented below:

Acid Digestion

Fish tissues were digested overnight in cold HCl and extracted with 25% dichloromethane in hexane (v/v). Entrained acid was neutralized with sodium bicarbonate and the extract reduced in volume by rotary evaporation. Volumes were adjusted

TABLE 1

MODEL COMPOUNDS AND SURROGATE STANDARDS

Class	Contaminant	Low Level Spike	High Level Spike	"Real" Sample
Volatiles	Trichlorobenzene	X	X	-
	Hexachlorobutadiene	X	X	-
	Hexachlorobenzene	X	X	-
Chlorophenols	2-Chlorophenol 3,4,5,6-D4*	-	-	X
	2,4 Dichorophenol	X	X	-
	2,4,5 Trichlorophenol	X	X	-
	Pentachlorophenol	X	X	-
Aromatic Amines	Diphenyl Amine	X	X	-
Organo chlorines & PCB	Mirex	X	X	-
	p,p'-DDE	X	X	-
	Dieldrin	X	X	-
	Aroclor 1242,54,60	X	X	-
Polyaromatic Hydrocarbons	Fluoranthene	X	X	-
	Phenanthrene D-10	X	X	-
	Anthracene D-10*	-	-	X

* Internal Standards

TABLE 2

SPIKING LEVELS OF FORTIFIED COMPOUNDS

FOR LOW AND HIGH LEVEL SPIKING OF "CLEAN" FISH HOMOGENATE

MODEL COMPOUNDS	Low Level Spikes ng/g	High Level Spikes ug/g
Dichlorophenol	147	26
Trichlorophenol	102	19
Pentachlorophenol	78	14
Diphenyl Amine	119	21
Trichlorobenzene	89	16
Hexachlorobutadiene	177	31
Hexachlorobenzene	110	20
Mirex	98	17
p,p-DDE	98	17
Dieldrin	98	17
Fluoranthene	124	22
Phenanthrene d10	39	7

to 10 mls 1:1 dichloromethane/cyclohexane. A 1 mL. portion was removed for lipid determination and a seven mL. aliquot was taken for GPC. The GPC eluate collected was rotary evaporated to approximately 2 mL., transferred to a reacti-vial with rinsings, with final volumes achieved by blowing down with a gentle stream of nitrogen.

Polytron Homogenization

Contaminants were extracted from the tissue using dichloromethane, sodium sulphate and a polytron tissue homogenizer. The dichloromethane extract was reduced in volume by rotary evaporation and made up to 10 mL. with 1:1 cyclohexane/dichloromethane in a calibrated centrifuge tube. A 1 mL. portion of the centrifuged extract was taken for lipid analysis with a 7 mL. aliquot of the remainder of the extract processed through gel permeation. Once again, the GPC eluate was rotary evaporated to 2 mL and quantitatively transferred to a reacti-vial where nitrogen gas was used to gently evaporate to final extract volume.

Steam Distillation

Fish homogenates were weighed and transfered to 50 mL round bottom flasks with rinses of organic-free water. The volume of water was adjusted to 300 mL. Three mL organic-free water and 10 mL hexane were charged into the condenser portion of the apparatus. Steam distillation of the samples continued for 3 hr from the time distillation began. At the end of this time heating was stopped and the system allowed to cool. The water and hexane was drained from the condenser into a centrifuge tube.

The organic layer was removed and reduced to the desired final volumn. The steam distillation extracts weree not processed through the GPC prior to GC/MS analysis.

The lipid removal step used throughout this study was by automated gel permeation chromatography (GPC) on Bio-beads SX-3 eluting with 1:1 dichloromethane:cyclohexane. Recovery studies were pereformed on the GPC at the same levels tested on the fish.

The GC/MS analyses were performed on a Finnigan 4510 using a 30 M SE-54 capillary column directly interfaced to the ion source. Electron impact (70 eV) spectra were obtained over the mass range of 90-550 A.M.U. scanning once per second. The column temperature program was 70^{o}C for 2 min., 70^{o}C to 280^{o}C at 10^{o}C per min. with a hold time of 10 min..

RESULTS AND DISCUSSION

Fortification Studies

GC/MS analysis of samples processed using the polytron homogenization procedure produced good mass spectra for all compounds for both the low level and high level spikes. The acid dissolution performed equally well for all compounds except diphenyl amine which would not be extractable from the acid digestion solution. Steam distillation failed to recover the higher chlorinated phenols, and resulted in very poor recoveries of diphenyl amine mirex, p,p'-DDE, dieldrin and fluoranthene. The recovery data for the three methods are reported in Table 3.

TABLE 3

RECOVERIES OF FORTIFIED COMPOUNDS

FOR LOW AND HIGH LEVEL SPIKING OF "CLEAN" FISH HOMOGENATE

MODEL COMPOUNDS	LOW LEVEL			HIGH LEVEL		
	Acid Digestion Recovery %	Polytron Homogenization Recovery %	Steam Distillation Recovery %	Acid Digestion Recovery %	Polytron Homogenization Recovery %	Steam Distillation Recovery %
Dichlorophenol	79.0	66.1	21.0	87.2	98.1	24.1
Trichlorophenol	78.3	63.0	-	78.9	64.3	-
Pentachlorophenol	105	100	-	81.7	40.0	-
Diphenyl Amine	-	36.5	47.0	-	83.8	14.0
Trichlorobenzene	90.7	74.4	49.0	76.6	83.8	61.4
Hexachlorobutadiene	64.7	68.6	40.0	70.1	83.3	62.6
Hexachlorobenzene	42.4	39.0	55.2	43.0	68.5	42.3
Mirex	36.8	44.7	4.5	83.1	94	3.5
p,p-DDE	*	*	*	94.7	93.0	9.9
Dieldrin	22.2	44.4	23.4	79.6	86.8	12.3
Fluoranthene	46.2	43.8	19.3	93.9	92.6	6.3
Phenanthrene d10	47.8	43.3	60.0	88.4	81.4	40.3

* High level in fish homogenate blank

Polychlorinated biphenyls (PCB) were also tested for recovery by the three methods. This data, reported in Table 4, shows that while both the acid digestion and polytron homogenization resulted in good recoveries for the dichloro- to heptachloro-biphenyl, isomers the steam distillation recoveries decreased significantly with increasing chlorination and the overall recovery of the PCB was less than 50%.

The results for the duplicate GPC spike recovery test are reported in Table 5. In all cases, the recoveries from the GPC were lower than the recoveries found in the overall fish fortification, extraction and GPC studies. This has been observed previously and seems to be related to the presence of lipid materials acting as a "keeper" in all extract concentration steps.

Figure 1 and 2 show the reconstructed ion chromatograms of the high level spike and low level spike respectively. From the RIC plots, the peaks of the model compounds are easily observed for the high level spike while the compounds are far less apparent in the low level spike samples. Foreground-background subtraction routines assist in further defining these lower concentration compounds and in providing useful mass spectra.

Naturally Contaminated Fish Study

The real fish homogenates demonstrated little variability in terms of contaminants extracted by polytron homogenization or acid digestion. As in the fortification study, the steam distillation extraction procedure was far less efficient in

TABLE 4

PCB RECOVERY

PCB Isomer	# of Isomers	Acid Digestion % Rec	Polytron Homogenization % Rec	Steam Distillation % Rec
Cl_2	3	74.3	65.5	91.8
Cl_3	4	85.4	70.5	73.1
Cl_4	4	93.5	85.7	35.9
Cl_5	5	94.0	92.7	19.3
Cl_6	6	102	103	4.7
Cl_7	4	103	103	-
Mean		92.0	86.7	37.5

Total PCB spike of 60 ug

TABLE 5

GPC SPIKE RECOVERIES FOR MODEL COMPOUNDS

MODEL COMPOUND	GPC Spike #1 %	GPC Spike #2 %	Average %
Dichlorophenol	78.5	67.0	72.8
Trichlorophenol	60.0	43.0	51.5
Pentachlorophenol	60.7	51.5	56.1
Diphenyl Amine	86.0	74.8	80.4
Trichlorobenzene	76.0	68.6	72.4
Hexachlorobutadiene	68.8	76.6	72.4
Hexachlorobenzene	74.5	70.3	72.4
Mirex	74.3	69.5	71.9
p,p'-DDE	95.0	72.5	83.4
Dieldrin	72.7	79.3	76.0
Fluoranthene	93.0	84.8	88.9
Phenanthrene d10	82.8	71.3	77.1

FIGURE - 1 RIC HIGH LEVEL SPIKE

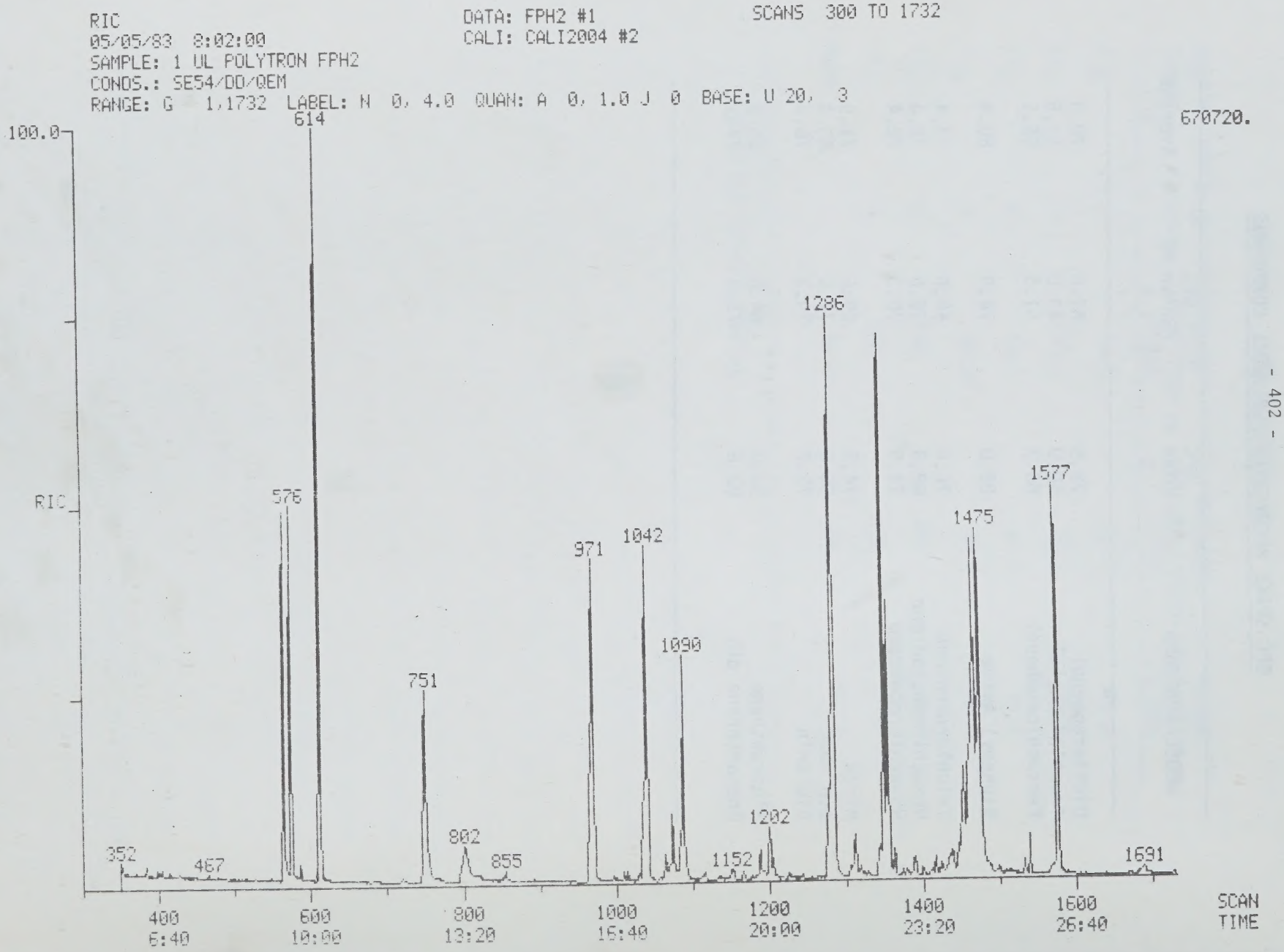
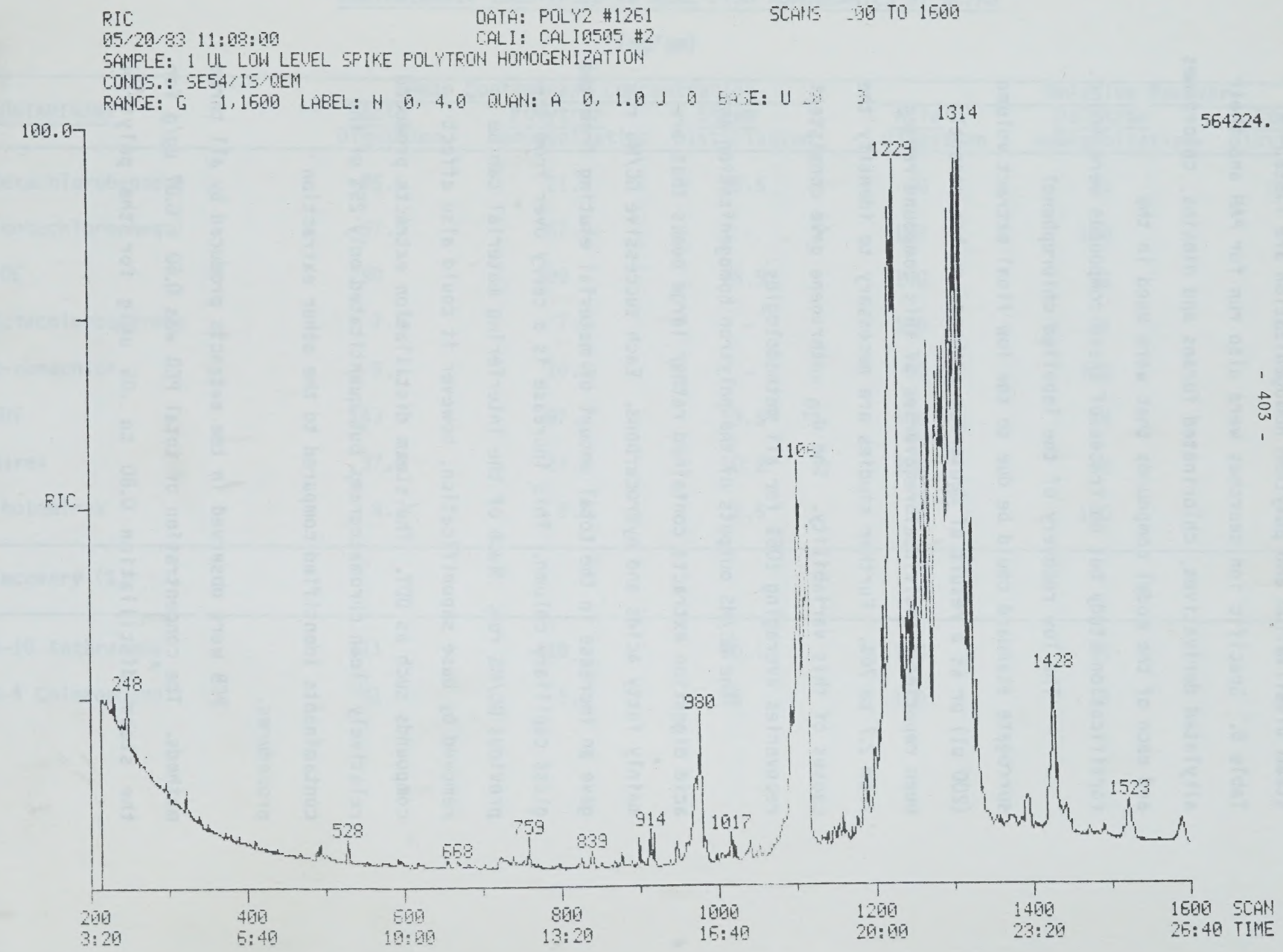


FIGURE - 2 RIC LOW LEVEL SPIKE



it's overall performance. GC/MS results from the acid digestion, steam distillation and polytron homogenization are listed in Table 6. Specific ion searches were also run for PAH and their alkylated derivatives, chlorinated furans and dioxins, chlordanes and each of the model compounds that were used in the fortification study but no traces of these compounds were found.

The low recovery of the labelled chlorophenol surrogate standard could be due to the low final extract volume (200 ul) or as a result of deuterium exchange as has recently been reported. Individual recoveries of this compound ranged from 2.7 to 76%. Further studies are necessary to identify the causes of this variability. The d_{10} anthracene gave consistent recoveries averaging 106% for all methodologies.

The GC/MS outputs of the polytron homogenization and acid digestion extracts contained rather large peaks that are mainly fatty acids and hydrocarbons. Each successive GC/MS run gave an increase in the total amount of material eluting from the glass capillary column. This increase is a carry over from the previous GC/MS run. Much of the interfering material can be removed by base saponification, however it could also affect compounds such as DDT. The steam distillation extracts produced relatively clean chromatograms but quantitated only 25% of the contaminants identified compared to the other extraction procedures.

PCB were observed in the extracts produced by all three methods. The concentration of total PCB was 0.60 $\pm$ 0.07 ug/g for the steam distillation 0.80 to .05 uglg for the polytron

TABLE 6

CONTAMINANTS IDENTIFIED IN REAL FISH HOMOGENATES BY GC/MS

(ng/gm)

CONTAMINANT	Mean Contaminant Concentration			Relative Recovery		
	Acid Digestion	Polytron Homogenization	Steam Distillation	Acid Digestion	Polytron Homogenization	Steam Distillation
Hexachlorobenzene	25.6	21.2	28.5	90	74	100
Pentachlorophenol	67.9	67.9	-	100	100	-
DDE	290	209	78.5	100	72	27
Octachlorostyrene	7.5	7.2	-	100	96	-
t-nonachlor	9.7	10.5	-	92	100	-
DDT	143	97.7	13.5	100	68	9
Mirex	37.0	33.9	10.2	100	92	28
Photomirex	8.7	8.8	2.5	99	100	28
Recovery (%)						
D-10 Anthracene	111	110	90.1	100	99	81
D-4 Chlorophenol	41	5.4	35	100	13	85

homogenization and 1.05 ± 0.06 ug/g for the acid digestion procedure.

CONCLUSIONS

The data obtained from the fortification studies and the analysis of naturally contaminated fish tissues show that the steam distillation technique is clearly inferior in the extraction and recovery of high boiling toxic organics from fish tissue samples. The acid digestion technique produced slightly higher recoveries of the chlorophenols, polycyclic aromatic hydrocarbons, volatile chlorinated hydrocarbons, and PCB in the fortification studies.

In addition, the acid digestion technique resulted in the higher contaminant concentrations for 75% of the compounds quantitated in the naturally contaminated tissues.

These results, along with the lower requirements in sample preparation time and equipment make the cold acid digestion technique the method of choice for the screening of Great Lakes sport fish for persistent and toxic organic compounds.

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The Development of a Freshwater Fish Test to Identify Aquatic Toxic Contaminants.

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Supported by the Ontario Ministry of the Environment as Provincial Lottery Project No. 64.

ABSTRACT

Determining the potential long and short term health effects of many of the chemicals being discovered in Ontario's receiving waters requires that new test approaches be developed. Gross genetic damage is a feature of many chemicals reacting with DNA, and in this study anaphase aberrations and micronuclei (indicative of chromosome damage) were assessed in fish embryo's. Brachydanio rerio (Zebrafish) embryo's were exposed to Ethyl methanesulphonate (EMS) for 24 hours. 1000 mg/L EMS is non-lethal to embryos over the 24 hour exposure period, however a reduction in developmental stage (growth) was seen in 100 and 1000 mg/L at 6 hours. A reduction in the mitotic index (total number of anaphases) was seen only in 1000 mg/L after 12 hours, and 10 mg/L after 24 hours. Exposure of Zebrafish embryo's to EMS resulted in an increase in anaphase aberration frequency, and in aberration severity, in as little as 6 hours when exposed to 100 mg/L or 24 hours when exposed to 1 mg/L. Micronuclei levels were insignificantly higher than control (due to wide variability) with 100 mg/L EMS, and further analysis of this approach's sensitivity is pending. It is apparent that both anaphase aberrations and micronuclei are sub-lethal phenomenon. A time and dose-dependant increase in embryo anaphase aberrations was present, and our experiments prove that this approach is as sensitive as more conventional approaches using adult fish. The impact of chemicals on somatic and germinal DNA is an important and growing area of study because these genotoxicants are linked to carcinogenesis, teratogenesis and mutagenesis. This test system provide's a means for detecting and assessing waterborne genotoxic chemicals at chemical levels below those affecting growth or survival.

INTRODUCTION

The increasing concern over aquatic contaminants with carcinogenic and mutagenic activity has prompted suggestions for using fish as sentinels (Beardmore et al., 1980; Alink, 1982). The monitoring of bioaccumulated chemicals in fish has been advocated (IJC, 1982), but not specifically for mutagenic and/or carcinogenic compounds. It may be more appropriate to look for the effects of mutagens/carcinogens rather than merely determining their presence. The potential impacts of aquatic mutagenic/carcinogenic compounds are very wide ranging, for both humans and the inhabitants of the water bodies affected (for review's see Alink, 1980; Klingerman, 1982). The most straight-forward approach is to determine that a change in DNA structure (mutation) has occured. The mounting evidence that somatic mutations may be a causative factor in carcinogenesis (Strauss, 1981) and that most carcinogens are mutagens (Ames and Hooper, 1978; Hart et al., 1977) has led investigators to study mutations arising in fish.

Work in detecting elevations in chromosome damage (Stromberg et al., 1981; Alink et al., 1980; Prein et al., 1978; Hooftman and Vink, 1981; Longwell and Hughes, 1980; Longwell 1981) has demonstrated that environmental contaminants can and do induce measurable chromosome damage in fish. The isolation of causative agents (mutagens and clastogens) requires that laboratory studies be performed either with the chemicals isolated from or preferably with the industrial effluents contributing to the area. While laboratory test systems using adult fish have been developed, chemical concentrations inducing significant effects normally do so only at very high or extended levels. Fish cell lines have been proposed (Beardmore et al., 1980) for increased sensitivity in screening aquatic contaminants, however whole organism assays would be preferable. The development and substantiation of an assay applicable to both laboratory and field work is the goal of this research program.

A variety of endpoints have been utilized in the past to assess the effects of mutagenic chemicals on fish, inluding sister-chromatid exchange (SCE), micronuclei (MN), and chromosome aberrations measured at both metaphase (MA) and anaphase (AA). While the SCE approach has gained wide acceptance, it requires that organisms be treated on two occasions with mutagenic chemicals, thus artificially increasing the background levels of mutation. The analysis of MA similarly requries colchicine, itself mutagenic, and both procedures are painstaking and time-consuming. The analysis of MN and AA do not utilize additional chemicals, do not increase the background rate of mutation, and appear's to

be the approach of choice. The sensitivity of any selected approach is of utmost importance, however little information is available to determine the relative sensitivity of the various endpoints, and this must then be explored before choosing a test.

The types of organisms which can be utilized is greatly affected by the chosen endpoint. Both the SCE approach and MA analysis require that organisms with a low number of large chromosomes be used, and this restricts the number of potential test organisms, especially for field work. While the mudminnow (Umbra limi) has proven to be useful in the field and laboratory, it is difficult to obtain large numbers of these fish. Because they must be obtained from the wild, mudminnow's are subject to background chemical burdens. The killifish (Nothobranchius rachowi) is now being utilized for laboratory work in Europe, it having the necessary chromosome complement for such studies, but it is expensive to raise and maintain because it has a short lifespan, is a limited spawner, and its eggs require a diapause to develop properly. An approach utilizing MN and/or AA would eliminate the neccesity for a low chromosome complement, be applicable to all species of fish, and would allow the use of the same approach in both the field and laboratory.

The search for a sensitive assay in aquatic toxicology has led to the use of embryo-larval stages, their sensitivity being comparable to that of chronic or life-cycle tests with juvenile and adult fish (McKim 1977; Macek and Sleight, 1977). The use of embryos to test pure compounds and effluents for mutagenicity may provide a sensitive whole organism test system in both the field and the laboratory. Their use in the field and laboratory has been investigated somewhat (Longwell and Hughes, 1980; Longwell, 1981; Hose et al., 1983) and they appear to be quite sensitive. The use of tropical fish embryo's in the laboratory has several advantages over cool or cold water fish: small eggs with relatively little yolk requiring higher temperatures allow for rapid development and a high number of mitotic figures; eggs can be exposed in small, static, non-aerated systems for a considerable period of time, and the fish are inexpensive and easy to raise and breed. This study provides evidence of fish embryo's response to a proven mutagen to provide information on the sensitivity and background values of an approach using AA and MN.

MATERIALS AND METHODS

Commercially obtained Brachydanio rerio (Zebrafish) adults 1.5 to 2 years old were held in activated carbon dechlorinated city of Toronto water in a 100 L glass aquarium with a 12/12 photoperiod and some natural lighting. The adults were fed Tetramin (TetraWerke Dr.rer.nat.Ulrich Baensch GmbH) staple flake food and frozen brine shrimp twice daily, and pairs were spawned in 10 L aquaria at 25 degrees C with aeration.

Beginning at the 2-64 cell stage, embryos were exposed to 20 ml test solution in 40 ml glass petri dishes at 25 degrees C. Ethyl methanesulphonate (EMS; Sigma) was diluted with dechlorinated water. Four separate tests were run, one for each concentration, with both control and exposed embryos (30 per concentration) fixed in buffered formalin at 6, 12 and 24 hours. Chemical concentrations tested were 1, 10, 100 and 1000 mg/L EMS.

Ten embryos were dechorionated, treated with 50% acetic acid for 15 minutes (after Longwell and Hughes, 1980) and then stained (5 minutes) and squashed in a solution of freshly filtered aceto-orcein containing 5% propionic acid. The edges of the slide coverslips were sealed to make a semi-permanent preparation. Randomly numbered slides from "normal" embryos were examined for aberrations in twenty late anaphase's per embryo (AA) at 1000X total magnification. Aberrations scored were principally chromatin bridges, acentric and attached fragments, lagging chromosomes and multipolar figures (Nichols et al., 1977;Kocan et al., 1982). Single chromatin bridges are indicative of an incomplete or asymmetrical exchange of DNA, leading to a thin strand of DNA being stretched between the dividing groups. Breaks in the chromatids or chromosomes are visible as unattached fragments at the equator (acentrics) or attached fragments which are joined to a group by a thin thread of chromatin.

Lagging chromosomes and multiple bridges are thought to be indicative of cellular toxicity resulting in sticky chromosomes (Longwell, 1978). Laggard chromosomes or attached and acentric fragments, which are left outside of the nucleus during the completion of division, form micronuclei, visible as small dark-staining bodies up to one-tenth the size of the normal nucleus. These were scored in the yolk-sac cells visible at 24 hours only, as insufficient numbers of yolk-sac cells are present at 6 or 12 hours. A normal embryo was classified as being normally developed grossly, possessing at least 10 anaphases microscopically and being without an excess of amorphous areas, consisting of denatured chromatin, a characteristic of moribundity (Longwell and Hughes, 1980). A limited number of

amorphous areas and pyknotic cells was considered normal, due to the resorbtion of embryonic tissues which were no longer necessary.

Data for up to 10 embryos from each time and concentration were analyzed for survival, total number of anaphases, number of anaphases with aberrations in the first 20 examined, and a developmental index, determined by assigning a number (based on Hisoaka and Battle (1957)) to each embryo and determining the average value. Micronuclei were analyzed at 24 hours only. Statistical analysis consisted of the Student's t-test at a 0.05 level of significance and ANOVA using the University of Guelph's APL Statistical library.

RESULTS

Control values

The design of this experiment facilitated a comparison of survival, AA and MN background frequency, developmental stage and number of anaphases for different groups of embryo's. The control fish featured a survival ranging from 96% (1000 mg/L) to 53% (1 and 10 mg/L), at 24 hours. All embryo's had 2 to 64 cells when the experiments were started, an age at which it is very difficult to remove the abnormally developing embryo's which are unlikely to survive.

The growth (developmental index) of the control groups was variable at all ages. To eliminate control variability, results for growth parameters are expressed as percentages of control values. The embryo's were mid-gastrulas at 6 hours, early embryo at 12 and mid-embryo at 24, corresponding to stages 15, 18 and 20 respectivly under the classification of Hisaoka and Battle (1958). The number of late anaphases was similar, only the 100 mg/L controls at 12 and 24 hours being different from another control group (1 mg/L) at the same age. Wide variability in the total number of anaphases was present, however the total number of anaphases in control embryos declined insignificantly from 31.2 at 6 hours to 28.1 at 12 and 27.8 at 24 hours.

AA background rates varied from 1.6/20 to 0.4/20 in groups of different parentage at different ages. The average background rate at 24 hours of age was 0.75/20, 0.9/20 at 12 hours and 1.53/20 at 6 hours of age. No relationship between control survival and anaphase aberrations was seen between groups, however the decrease from 1.53/20 at 6 hours to 0.9/20 AA's at 12 hours corresponded with the general finding that mortalites were first evident at 12 hours. In only one instance was any control anaphase aberration rate significantly different at the same stage from another (1000 mg/L control versus 10 mg/L control at 6 hours). Background rates of yolk-sac MN at 24 hours of age varied from 0.32% to 1.6%. MN rates appeared somewhat to be related to survival, as the 1 and 10 mg/L controls, which had 50% control survival, had the highest MN values (1.6% and 0.87% respectivly). None of the control MN frequencies were significantly different. Based on the aforementioned findings, all control embryos regardless of parentage featured similar background levels of AA's, MN and growth indicators while survival variability appeared to be due to parental differences in two groups.

Survival:treated embryo's

The survival of EMS treated embryo's was only significantly different from control in one tested group, the 100 mg/L group at 24 hours, a reduction by 50% (Figure 1). This was an anomaly, as no significant difference in survival was evident between control and treated groups at 12 hours in 100 mg/L EMS, or at any time in 1000 mg/L. Subsequent testing with EMS has found that the 24 hour LC50 is in excess of 1000 mg/L and repeated experiments with both embryo's and juveniles has yielded little 24 hour mortality in EMS concentrations up to 1000 mg/L. Analysis of the trends in survival of treated embryo's versus controls (Figure 1) reveals that at all times 1 and 10 mg/L EMS had no significant effect. 1000 mg/L and 100 mg/L EMS consistently (5 of 6 concentration/time combinations) yielded relative survival only slightly less that 100% (an average of 87% survival). The onset of mortality in both control and exposed fish was generally at 12 hours, very little mortality being seen at 6 hours. EMS is non-lethal at concentrations up to 1000 mg/L for 24 hours.

Growth and division rate:treated embryo's

The greatest effect on growth was apparently felt at 6 hours (Figure's 2A,B) as the 1000 and 100 mg/L concentration's reduced the developmental stage to 80% and 90% of control respectivly. This delay apparently was temporary as by 12 hours all concentrations were reduced to only 95 to 98% of control. At 24 hours an apparent concentration dependant depression of developmental stage was again visible (Figure 2A), and this reduction of growth by 5 and 10% in 100 and 1000 mg/L was significant. It appears that the gastrula stage (6 hours) and mid-embryo (24 hours) stages are somewhat more susceptable than the early embryos (12 hours) to the toxicant effects of EMS (Figure 2B). With the exception of 100 and 1000 mg/L at 6 hours, it was felt that the reduction in growth (developmental stage) was of little consequence to the embryo.

As a second measure of growth rate (mitotic index) the total number of anaphases per embryo was examined (Figure 3). The effect of EMS appears to be a concentration-dependant reduction in the number of anaphases below control levels, in 10 mg/L EMS and above at 24 hours and in 1000 mg/L alone at 12 hours. The pattern at 6 hours is highly variable. The effect seen in the 100 mg/L EMS embryo's at 6 hours coincides with the reduction in devlopmental stage seen in the sensitive gastrula stage (Figure 2). The absence of an effect in 1000 mg/L is unexplained. It may have been that these embryo's had their growth slowed so much as to not have

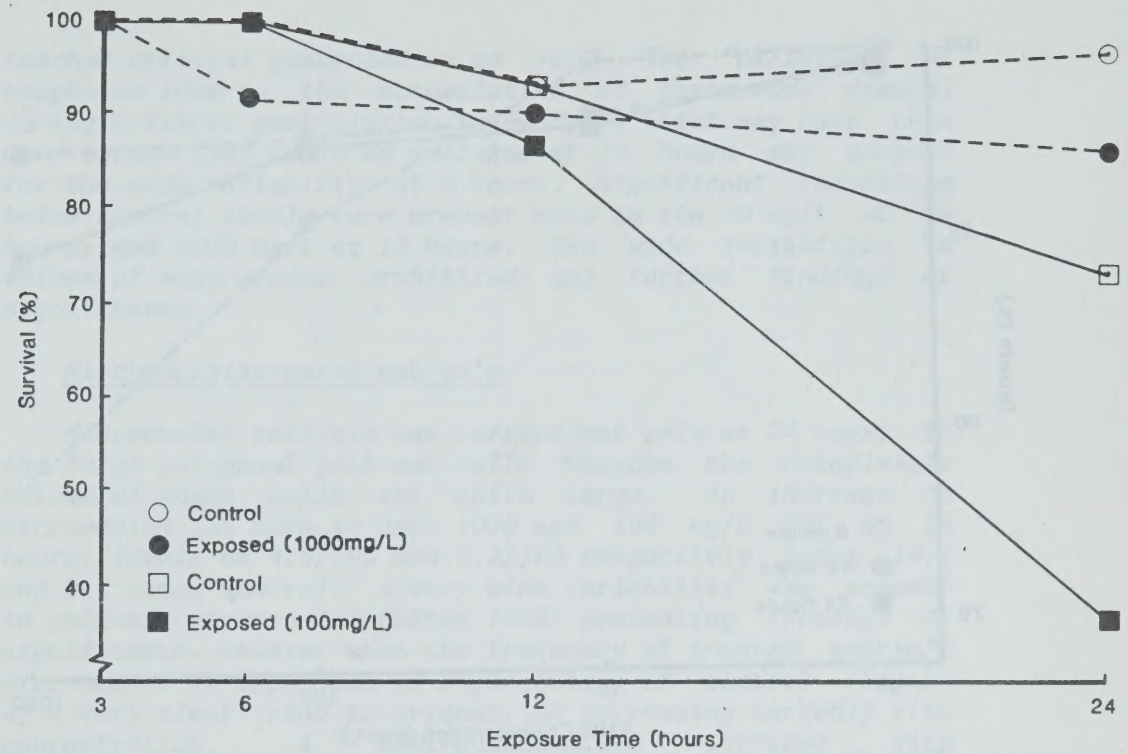

Figure 1A : Survival of Brachydanio rerio embryos exposed to 100 and 1000 mg/L Ethyl methanesulphonate and unexposed paired controls for up to 24 hours.

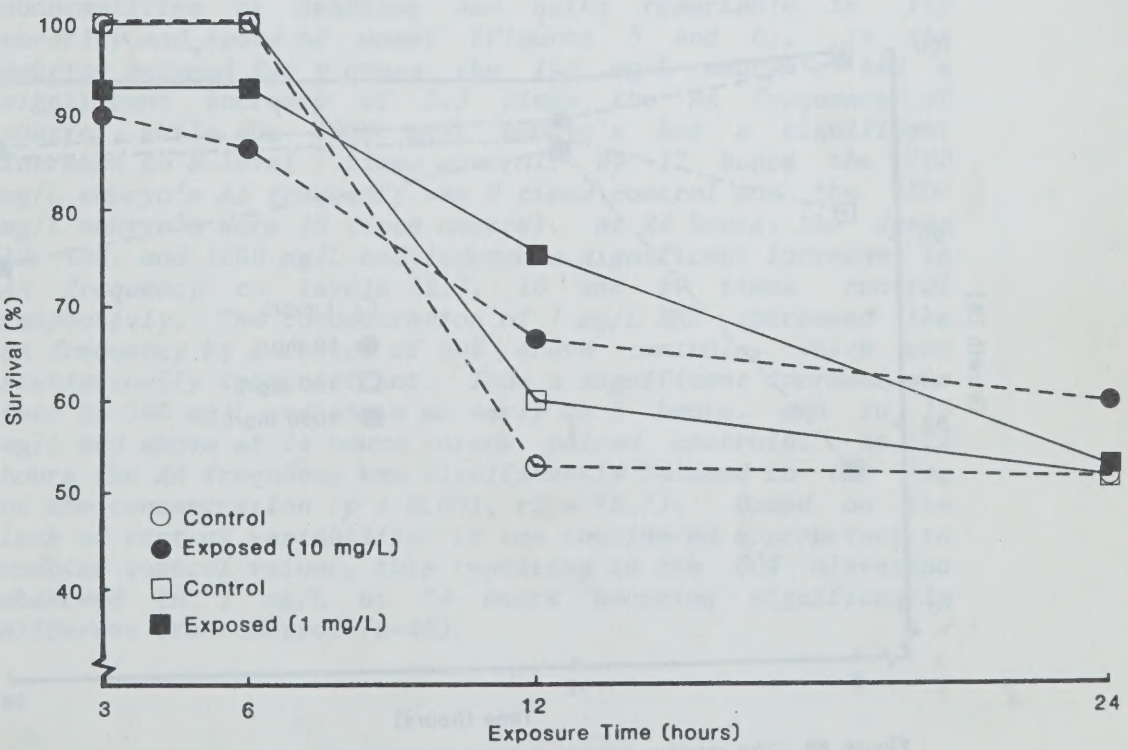

Figure 1B : Survival of Brachydanio rerio embryos to 1 and 10 mg/L Ethyl methanesulphonate and unexposed paired controls for up to 24 hours.

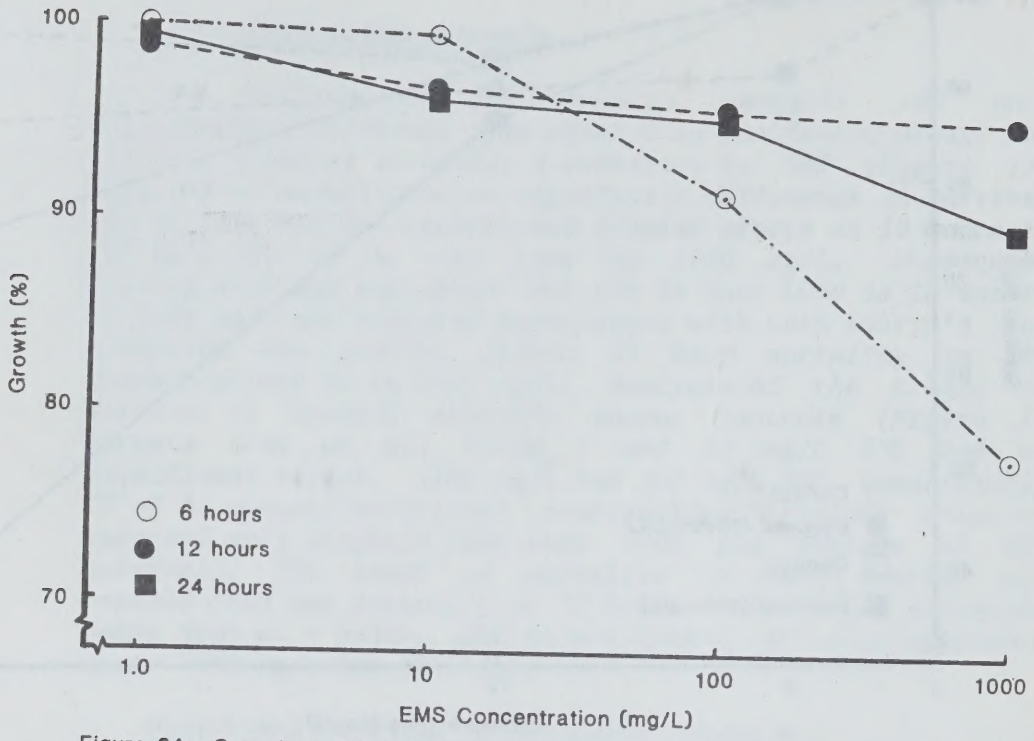

Figure 2A : Growth of Ethyl methanesulphonate exposed Brachydanio rerio embryos after 6, 12 and 24 hours. Growth is expressed as the ratio (percent) of the exposed embryos quantitative developmental index to the controls (unexposed embryos) thus indicating the relative reduction of developmental stage only.

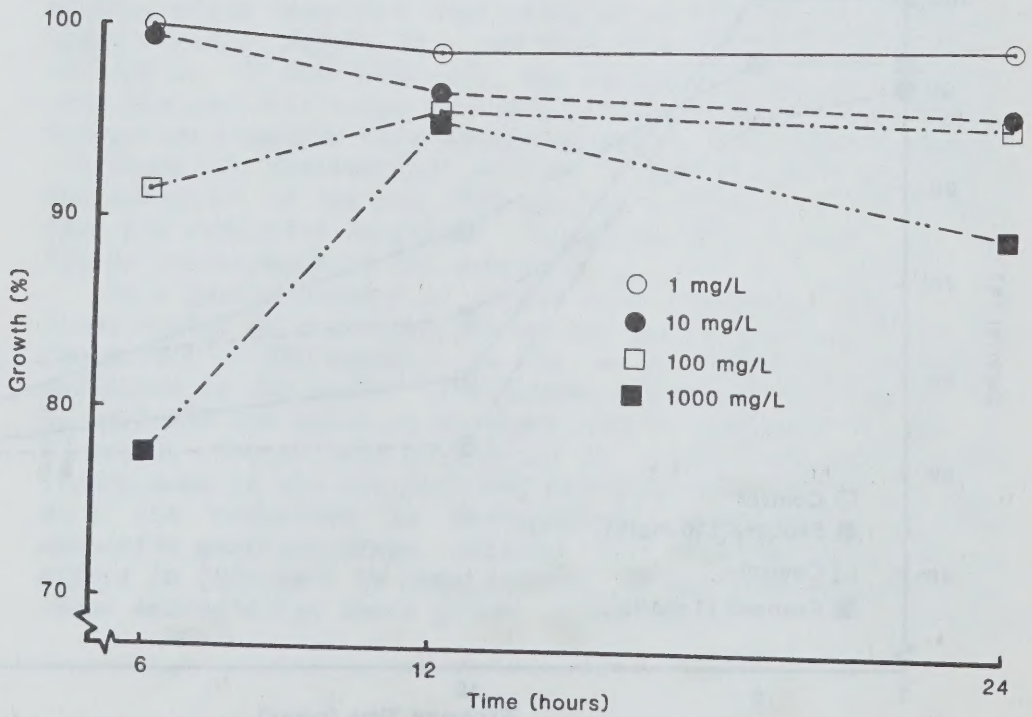

Figure 2B : The relative growth of Ethyl methanesulphonate exposed Brachydanio rerio embryos expressed as a ratio (percentage) to their paired controls at 6, 12, and 24 hours.

reached critical gastrulation as yet. The "piling-up" of anaphases (due to the accumulation of chromosome damage) during critical gastrulation in embryo's which may have then died before they could be analyzed at 12 hours may account for the wide variability at 6 hours. Significant reductions below control levels were present only in the 10 mg/L at 24 hours, and 1000 mg/L at 12 hours. The wide variability in values of many groups prohibited any further findings of significance.

Micronuclei:treated embryo's

Micronuclei analysis was carried out only at 24 hours in the large polygonal yolk-sac cells because the cytoplasmic volume of these cells was quite large. An increase in micronuclei was seen in both 1000 and 100 mg/L EMS at 24 hours, levels of 4.5/100 and 2.2/100 respectivly being 14.2 and 5.1 times control. A very wide variability was present in yolk-sac micronuclei (often 150%) preventing findings of significance, however when the frequency of treated embryo's micronuclei is expressed as a percentage of control (Figure 4) a very clear trend is evident, MN increasing markedly with concentration. A semi-quantitative increase with concentration was seen in the number of pyknotic (dead) cells, not unlike micronuclei.

Anaphase Aberrations:treated embryo's

The effect of EMS on the total number of chromosomal abnormalities at anaphase was quite remarkable in its severity and speed of onset (Figures 5 and 6). In the embryos exposed for 6 hours the 100 mg/L embryo's had a significant increase of 2.5 times the AA frequency of control, while the 1000 mg/L embryo's had a significant increase to a level 7 times control. By 12 hours the 100 mg/L embryo's AA frequency was 9 times control and the 1000 mg/L embryo's were 10 times control. At 24 hours, the doses 10, 100, and 1000 mg/L had induced a significant increase in AA frequency to levels 2.3, 10 and 29 times control respectivly. The concentration of 1 mg/L EMS increased the AA frequency by a factor of 60% above controls, which was statistically insignificant. Thus a significant increase was seen in 100 mg/L and above as early as 6 hours, and in 10 mg/L and above at 24 hours versus paired controls. At 24 hours the AA frequency was significantly related to the log of the concentration ($p < 0.001$, $r2 = 78.7$). Based on the lack of control variability, it was considered appropriate to combine control values, this resulting in the 60% elevation observed in 1 mg/L at 24 hours becoming significantly different from control (n=40).

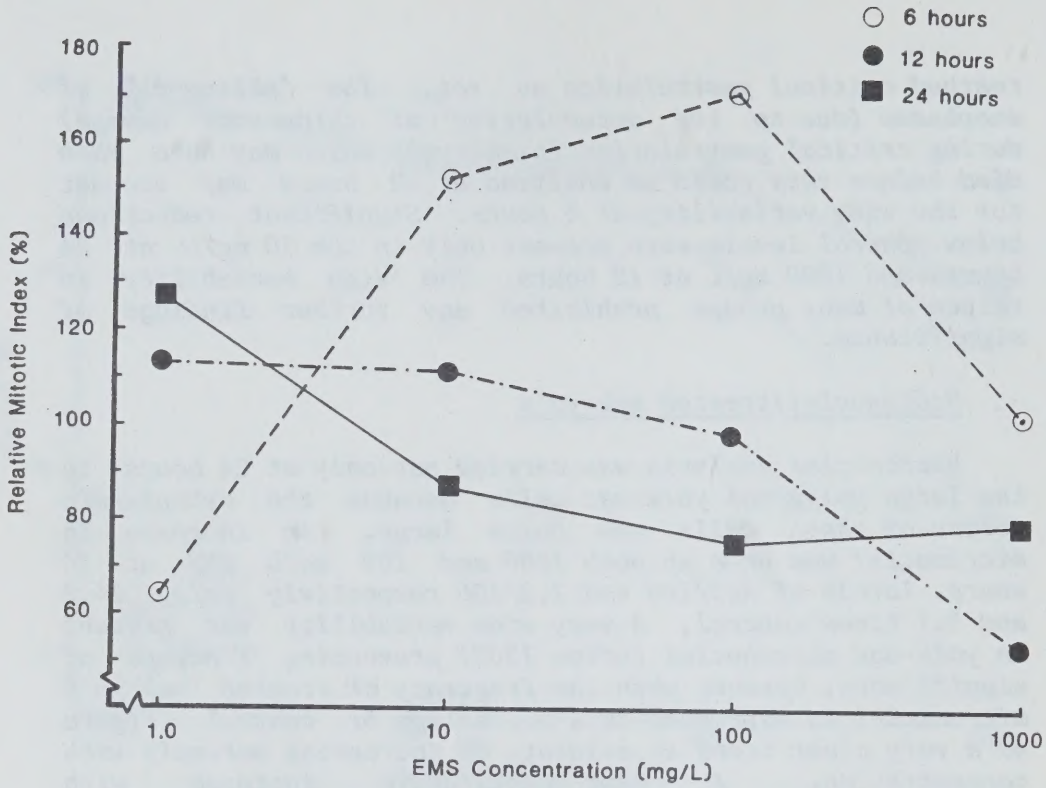

Figure 3 : Fluctuations in the relative mitotic index (the ratio (%) of the no. of anaphases in exposed embryos versus the no. in control) in Brachydanio rerio embryos exposed to Ethyl methanesulphonate for 6, 12, and 24 hours.

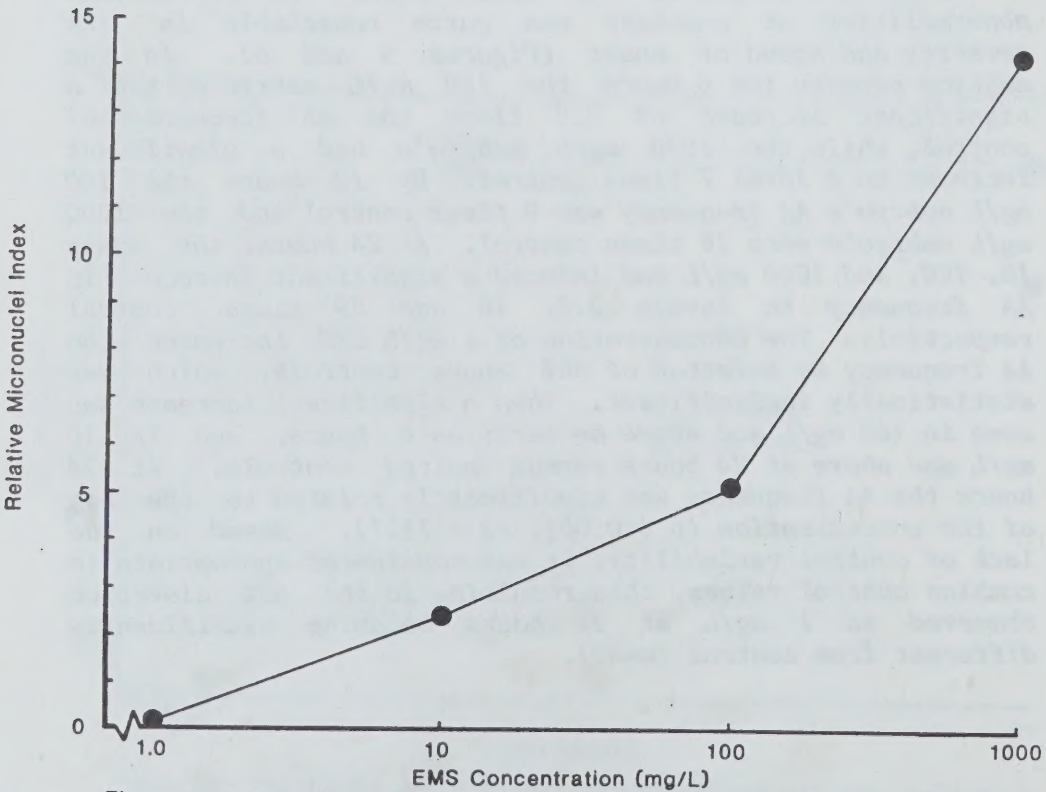

Figure 4 : The influence of Ethyl methanesulphonate on the abundance of yolk-sac micronuclei in Brachydanio rerio embryos exposed for 24 hours. The number of micronuclei in the exposed embryos divided by the number in the control embryos forms the Relative Micronuclei Index.

An analysis of the change in AA rate over time (Figure 6) reveals that the EMS concentrations of 1, 10 and 100 mg/L induce generally more aberrant anaphases with time, up to 24 hours. The 1000 mg/L EMS appears to have levelled off at 12 hours, with 77% of the anaphases present having aberrations.

The types of defects induced by EMS were primarily attached and acentric fragments, lagging chromosomes and chromatid bridges (single), accounting for 94% of the observed abnormalities at 24 hours (Table 1). The pattern is quite different at 6 and 12 hours as at these times multiple abnormalities (generally a combination of multiple bridges or bridges with another type of defect) were the largest single type of damage, followed by fragments, bridges and lagging chromosomes. The elevated incidence of multiple defects at 6 and 12 hours may reflect increased sensitivity at the cell level to the toxic effects of EMS.

The ratio of several of the types of damage to each other yields some insight into the action of EMS. Comparison of the ratio of either of the two types of fragments (acentric and attached) to lagging chromosomes in all times reveals that the ratio increases markedly with concentration. This is also the case when comparing the ratio of fragments to bridges in the 6 and 24 hour exposed embryo's, and would hold true in the 12 hour embryo's except for the large increase of multiple defects, reducing the apparent incidence of single bridges artificially, as most multiple defects included bridges. It would appear that attached and acentric fragments are the primary damage caused by EMS, the bridges and lagging chromosomes being secondary damage.

Table 1A: The anaphase abnormalities in control and exposed embryos at 6 hours after the initiation of the experiment. Control embryo's are pooled at each time. All values are expressed relative to the number of anaphases examined for defects, normally 20 in each embryo.

Dose	Number of Embryo's	% Normal	% Attached Fragments	% Acentric Fragments	% Chromatin Bridges	% Lagging Chromosomes	% Side-arm Bridges	% Multipolar Figures	% Multiple Defects	Others
0	25	94.5	0.0	0.5	1.8	2.7	0.0	0.0	0.4	0.0
1	6	96.6	0.0	0.8	0.8	1.7	0.0	0.0	0.0	0.0
10	7	94.3	0.0	0.0	0.7	3.5	0.0	0.0	1.4	0.0
100	10	84.0	0.0	1.5	5.5	4.5	0.0	0.0	4.5	0.0
1000	10	46.3	2.6	12.1	11.1	13.7	0.0	0.5	14.2	0.0

Table 1B: As for Table 1A except that values are for embryo's after 12 hours.

Dose	Number of Embryo's	% Normal	% Attached Fragments	% Acentric Fragments	% Chromatin Bridges	% Lagging Chromosomes	% Side-arm Bridges	% Multipolar Figures	% Multiple Defects	Others
0	37	96.5	0.0	0.1	0.6	2.7	0.0	0.0	0.0	0.0
1	10	97.9	0.0	0.0	1.0	1.0	0.0	0.0	0.0	0.0
10	10	95.4	0.5	0.0	0.5	3.5	0.0	0.0	0.0	0.0
100	10	79.0	0.5	7.5	3.5	7.5	0.0	0.5	1.5	0.0
1000	7	22.5	0.9	13.5	2.7	14.4	0.0	0.0	46.8	0.0

Table 1C: As for Tables 1A and 1B except that values are for embryos after 24 hours.

Dose	Number of Embryo's	% Normal	% Attached Fragments	% Acentric Fragments	% Chromatin Bridges	% Lagging Chromosomes	% Side-arm Bridges	% Multipolar Figures	% Multiple Defects	Others
0	40	95.3	0.4	1.0	2.1	0.6	0.1	0.0	0.0	0.4
1	10	91.7	0.8	1.6	2.7	2.4	0.0	0.0	0.0	0.8
10	10	90.4	0.5	0.9	2.7	2.7	0.5	0.9	0.5	0.9
100	10	61.9	2.3	10.0	5.4	14.6	0.7	1.9	2.7	0.4
1000	10	33.5	7.9	21.9	15.9	16.5	0.0	1.2	3.0	0.0

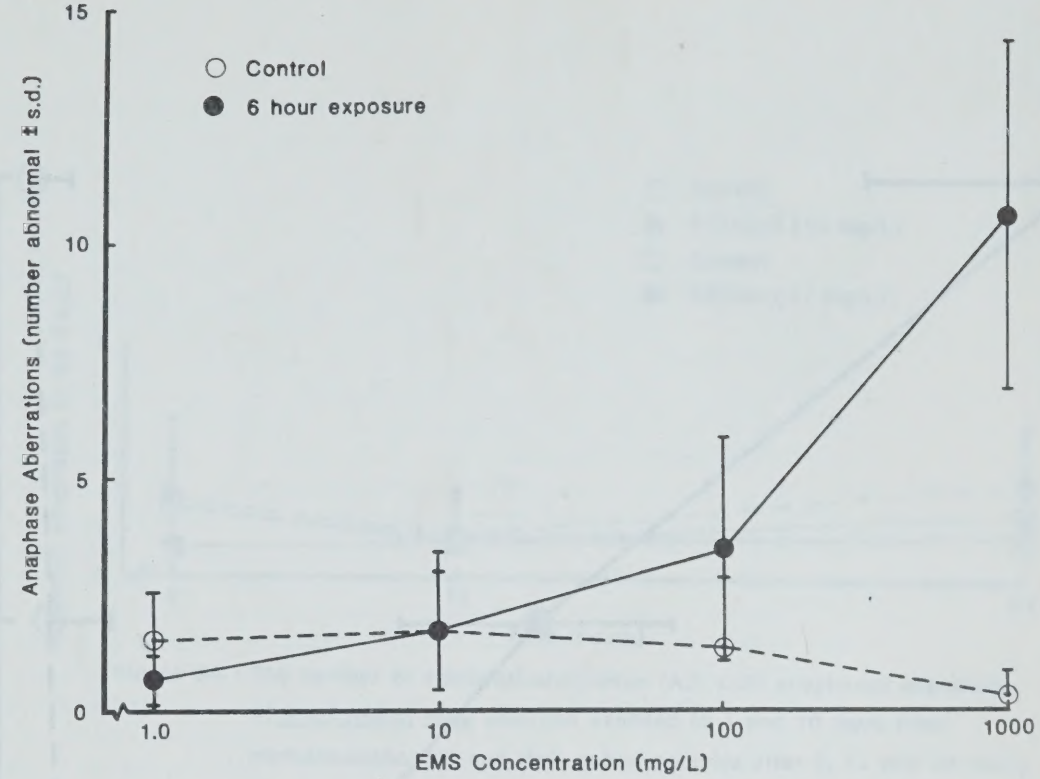

Figure 5A : The genotoxicity of Ethyl methanesulphonate in Brachydanio rerio embryos exposed for 6 hours. Genotoxicity is measured as the number of A.A.'s in 20 anaphases examined per embryo.

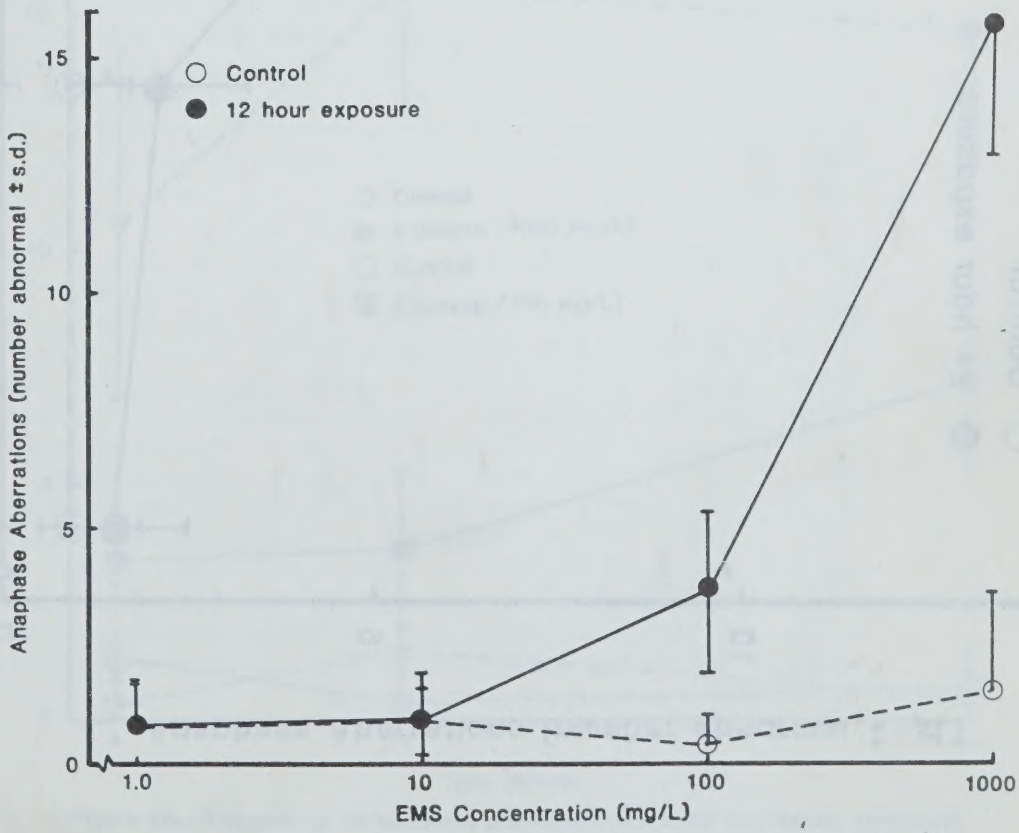

Figure 5B : The genotoxicity of Ethyl methanesulphonate in Brachydanio rerio embryos exposed for 12 hours.

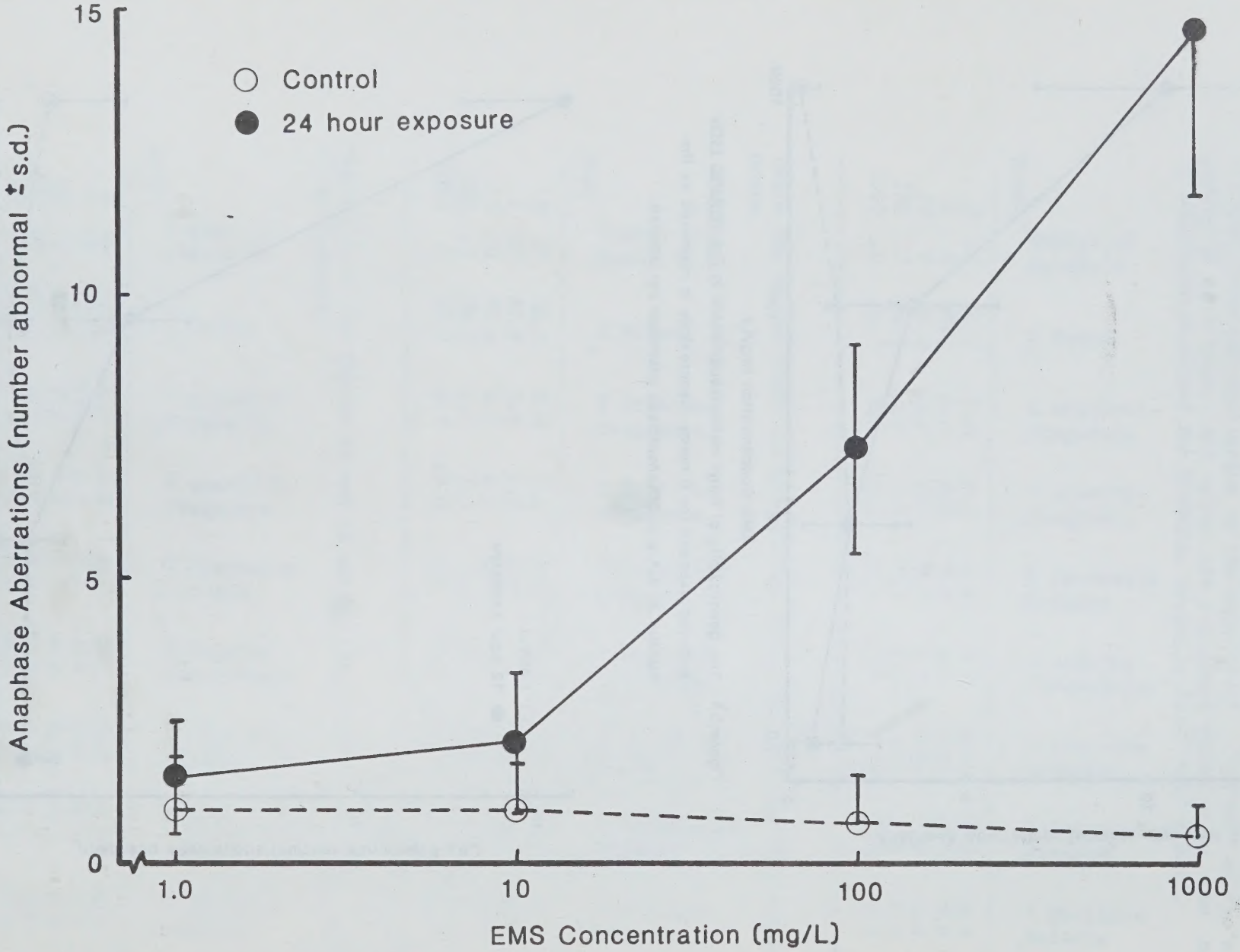

Figure 5C : The genotoxicity of Ethyl methanesulphonate in Brachydanio rerio embryos exposed for 24 hours.

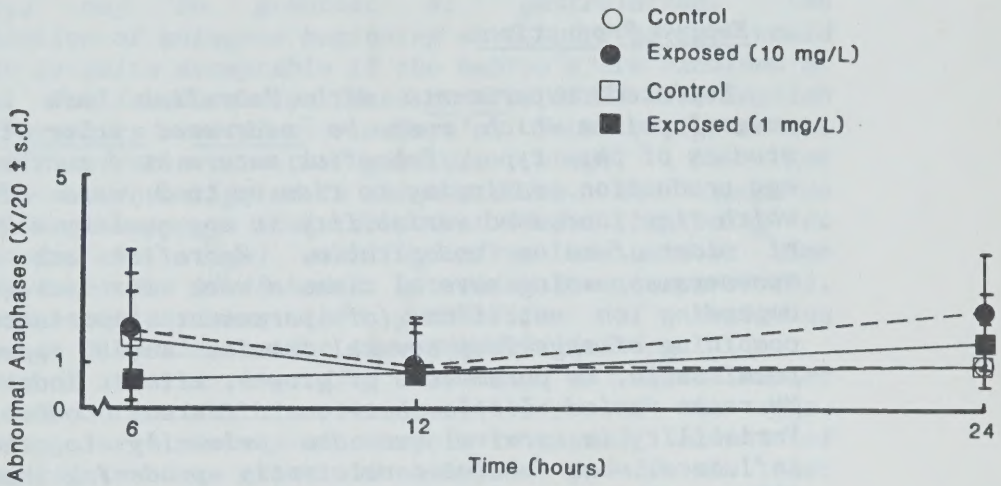

Figure 6A : The number of abnormal anaphases (A.A.'s/20 anaphases examined) in Brachydanio rerio embryos exposed to 1 and 10 mg/L Ethyl methanesulphonate and their paired controls after 6, 12 and 24 hours.

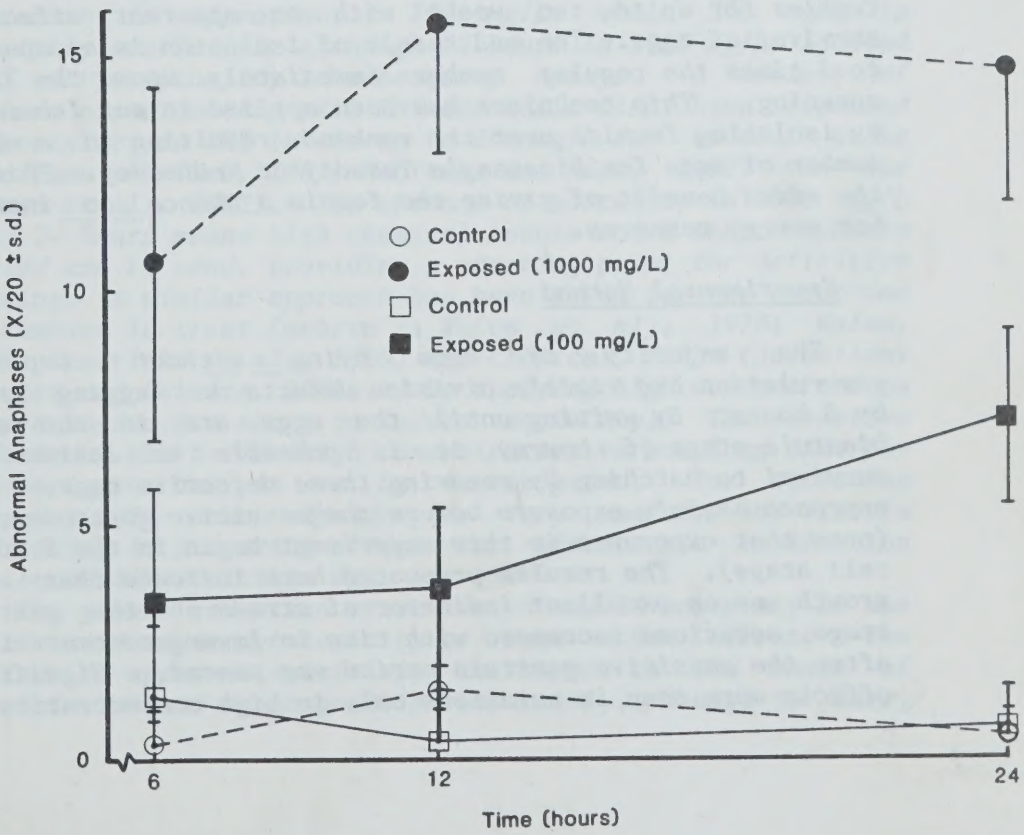

Figure 6B : The number of abnormal anaphases (A.A.'s/20 anaphases examined) in Brachydanio rerio embryos exposed to 100 and 1000 mg/L Ethyl methanesulphonate and their paired controls after 6, 12 and 24 hours.

DISCUSSION

Embryo Production

Repeated experiments with Zebrafish have highlighted several points which must be addressed prior to further studies of this type. Zebrafish mature at 4 months of age, egg production continuing to rise up to 2 years of age, at which time increased variability in egg quality makes the use of older females undesirable. Zebrafish are fractional spawners, spawning several times a week for several weeks, depending on nutrition (of paramount importance). The combining of eggs from several females would appear to be permissable, as parameters of growth, mitotic index, AA and MN rates varied little between different control groups. Variability in survival was due primarily to the females influence, some females consistently producing spawns with 95% survival, other's consistently producing 5% survival. By spawning a female several times and recording survival, it is an easy task to accumulate several females with excellent spawning success. The availability of a large number of healthy eggs continuously for up to 3 weeks at a time can then be assured.

Over-ripeness appear's to be a problem if females are isolated for 15 days to a month (Niimi and La Ham, 1974; Laale, 1977) however our laboratory has successfully isolated females for up to two weeks with no apparent effect on survival of eggs. The end result of isolation is a spawn 2 to 3 times the regular number immediately upon the first spawning. This technique has been applied in our laboratory by isolating females over the weekend, resulting in a large number of eggs for bioassay's Tuesday or Wednesday. This has the added benefit of giving the female a chance to increase her energy reserves.

Experimental Method

The majority of eggs dying without completing gastrulation had visible division defects or lagging growth by 6 hours. By waiting until the eggs are in the early blastula stage (6 hours) it is possible to achieve 95% survival to hatching by removing these defective eggs. This approach begin's exposure before the sensitive gastrula stage (note that exposures in this experiment began in the 2 to 64 cell stage). The results presented here indicate that while growth was an excellent indicator of stress at the gastrula stage, mutations increased with time in lower concentrations after the sensitive gastrula period was passed. Significant effects were seen in mutations only in high concentrations at

the gastrula stage. While sensitivity to maternally transferred mutagens (yolk) or paternal effects (ie. dominant lethality) may be greatest at gastrulation, the administration of mutagens beginning at 6 hours of age would appear to be quite acceptable if the embryo's are examined at 24 hours. Published studies (Hose et al., 1983) with English sole (Parophyrs vetulus) embryo's found significant cytogenetic effects at the gastrula stage. No time dependancy was investigated in these studies, and exposures utilized Benzo-a-pyrene administered at 11.5oC (beginning at the blastula cap stage) sampling after an unknown time (propably in excess of 48 hours at this low temperature). This work substantiates the possibility of beginning exposures at blastula, rather than at fertilization.

A note of interest regarding the use of Zebrafish as mutagen test organisms is the small size of the eggs, facilitating large numbers of embryo's in a small amount of test chemical (we have grown 50 embryo's in 20 ml. test solution for up to 4 days with no apparent ill effects) without replacement. Small amounts of test chemical can be used with eggs, as opposed to experiments with adult fish, requiring large amounts of test chemical.

Health effects

The absence of EMS induced mortality in this experiment was to be expected. The 96 hour LC50 of EMS with juvenile Zebrafish is approximatly 750 mg/L, while 250 mg/L is completly non-lethal, although 72 hours in 1000 mg/L induced 100% embryo lethality in subsequent assays. A variety of authors experimenting with EMS and adult fish have reported no mortality in EMS levels up to 200 mg/L for several weeks (Hooftman and Vink, 1981; Hooftman and Raat, 1982; van der Hoeven et al., 1982). The absence of mortality in 1000 mg/L over 24 hours means high chemical levels and a short exposure period can be used, providing a starting point for definitive testing. A similar approach has been taken for the induction of tumours in trout (embryo's; Wales et al., 1978; Wales, 1979; Hendricks et al., 1980) and tropical fish (juveniles; Schultz and Schultz, 1982a,b) by applying high concentrations over a period too short to induce mortality. The relative effects of short time/high concentration versus long time/low concentration has yet to be investigated. For screening purposes a 24 hour exposure is very useful, and little difficulty in preparing slides for microscopic examination was encountered at this age.

The growth reduction evident at 6 and 24 hours may be due to tissue damage, cytotoxicity or some other effect. The periods of apparent sensitivity correspond to the onset of gastrulation and organogenesis respectivly. Damage to early

"primitive" tissues could severly delay further development, other cell types being forced to differentiate, replacing the damaged ones. Subsequent studies performed with EMS have shown the growth reduction caused by 24 hours exposure to EMS is not recovered, even if the embryo's are removed to clean water, for the period up to hatching (4 days). The use of the number of anaphases as a mitotic index shows a tissue-level growth reduction at 12 hours in 1000 mg/L EMS and above 10 mg/L at 24 hours. This indicates that the ability of individual cells to divide normally corresponds directly to the growth delay seen in 24 hours as reduced developmental stages. The absence of a growth delay at 12 hours probably reflects the death of moribund embryo's between 6 and 12 hours.

A decrease in spontaneous (background) AA frequency over time was seen in this study corresponding to the onset of mortality in all groups between 6 and 12 hours. Similarly, a decrease in AA's after gastrulation in Atlantic mackeral (Scomber scombrus) corresponded with an increase in the percentage of non-moribund and live embryo's sampled (Longwell, 1978). Both studies support the contention that many embryo's or cells with abnormal divisions don't complete gastrulation (A.C. Longwell, pers. comm.). The level of abnormal anaphases in control fish (approximatly 7.6%,4.5% and 3.7% at 6,12 and 24 hours of age) was similar to those found for English sole (4.3%; Hose et al., 1982) and mackeral (5.9% across a range of ocean samples; Longwell and Hughes, 1980). These values are much lower than the 12% found in a Rainbow trout (Salmo gairdneri) gonad cell line (Kocan et al., 1982).

The yolk-sac micronuclei found in this study (the numbers were insignificantly higher than control) supports the use of this approach with fish. Hooftman and Raat (1982) have been able to induce a statistically significant dose-dependant increase in micronuclei in adult fish with EMS. Other authors using fish and sea-urchin embryo's (Hose et al., 1982,1983), while finding an increase apparently were unable to generate a dose response or a significantly increased micronucleation rate. A possible problem with micronucleus use in embryo's is related to the natural process of apoptosis taking place (see Wyllie et al., 1980 for a review) which results in bodies very similar to micronuclei being phagocytozed by surrounding tissues. If a tissue is undergoing normal "degeneration" and subsequent apoptosis (embryo's would for example remove phylogenetic vestiges and metamorphose through apoptosis) it would be difficult to seperate this from the basophilic necrotic nuclear debris resulting from cytotoxicity. It has been suggested (see the review by Wyllie) that apoptosis is the mechanism by which proliferating tissues eliminate cells with genetic

abnormalities (mutations); regardless micronuclei may be confused with apoptotic bodies either "normal" or "mutant" in origin, or phagocytozed necrotic debris. Further investigations are planned to further assess the relative efficiency of the micronucleus test in embryo's, and its relation to apoptosis.

The usefullness of Zebrafish embryo's for assessing waterborne mutagens appears to be very good. It is apparent that both a time and concentration dependancy are present in AA analysis. In lower concentrations (1 to 100 mg/L) the amount of damage increases with time until 24 hours, perhaps continuing to rise until some plateau is reached. This plateau was reached in 1000 mg/L after 12 hours. A longer exposure may make this test more sensitive to lower chemical levels for two reasons. Bio-accumulated chemical burdens will increase with time until a steady state is reached; and secondly, some types of damage require several rounds of replication before they become apparent, or are "fixed", while others dissapear through cell death. While micronuclei abundance was too variable for statistical significance to be found in this study, a study with the mudminnow (Hooftman and Raat, 1982) found an increase in peripheral blood micronuclei with time. This increase was due to the two factors previously described, the rise in numbers of micronucleated erythrocytes occuring because this damage type is quite persistent. In contrast to MN, the examination of AA's represents a single point-in-time measure of damage, damage which becomes invisible when mitosis is aborted, or becomes fixed, either as micronuclei, or some viable mutation in the daughter cell.

The absolute sensitivity of this test approach is excellent, based on the findings of a significant increase in 24 hours of AA's in 10 mg/L EMS with paired (n=10) controls, or in 1 mg/L EMS with grouped (n=40) controls. This level of sensitivity can be compared to published work. Studies with other species of adult fish have detected significant increases in genetic damage at levels of 8 mg/L EMS (killifish) after a 48 hour exposure (Hooftman, 1981), 40 mg/L (mudminnow) for 48 hours (Hooftman and Vink, 1981) and 50 hours (killifish) or 120 hours (mudminnow) in 24 mg/L EMS (van der Hoevan et al., 1982). In these cases, measurments of genetic damage were made in classically accepted fish species, with a small number of large chromosomes, measuring damage as aberrations or SCE's at metaphase. While it has been suggested (Klingerman, 1982) that these approaches are the most suitable and sensitive for use with piscine subjects, this study and others (Kocan et al., 1982; Hose et al., 1982,1983) found that the efficiency and sensitivity of AA analysis makes it preferable. The sampling of resident populations has been performed with embryo analysis which has

further provided an indication of the possible sensitivity of embryo's and this approach (Longwell and Hughes, 1980).

The use of embryo's should conceivably make the analysis of AA's more sensitive than with adult fish, because of the inherent sensitivity of the embryo and the rapid mitotic division providing a large number of anaphases for analysis. A comparison can be made for English sole exposed to Benzo-a-pyrene via two approaches. Juvenile sole (30 gm.) injected intraperitoneally with 0.05 ug/g (50 ppb) had a significantly increased SCE level at day 7 after injections on days 0 and 3 (Stromberg et al., 1981) while sole embryo's exposed to 2.1 ug/L (ppb) had a significantly higher AA rate than control as gastrula's (Hose et al., 1983). While the exposure methods are dissimilar the sensitivity of the embryo AA analysis is apparent. In a study with sea urchins, Hose et al. (1982) found additionally that AA's were significantly higher in chemical concentrations well below those causing teratogenesis. The speed and ease of analysis of AA's and perhaps micronuclei make this approach the most sensible and cost/time efficient.

Some of the types of AA's seen in this study are presumed to be cyto-toxic (lethal to the cell) to the immediate daughter cells of the dividing parent (Longwell, 1978). Two of the 4 major types of damage found in this study (attached and acentric fragments) are consistent with the alkylating properties of EMS, causing breaks in the DNA sequence. The gaps and breaks observed by other researchers at metaphase result in acentric and attached fragments at anaphase (Nichols et al., 1977), eventually resulting in micronuclei (Schmid, 1975; Kocan et al.,1982). Single bridges, lagging chromosomes and multipolar spindles result in abnormal numerical distribution of chromosomes. It has been stated (Longwell, 1978) that multiple bridges are a toxic phenomenon, however their induction by EMS raises question's about this. The finding that multiple bridges are accompanied by damage regarded as truly genotoxic (acentric and attached fragments) appears to indicate that they really are cytogenetic in origin. Any event potentially affecting DNA distribution, structure or functional capability must be regarded as mutagenic in character, regardless of whether such damage is direct (interaction with DNA) or indirect (interaction with proteins of either the replication or repair mechanism).

The significance of the genotoxic (mutagenic) damage seen in dividing embryo cells includes the immediate death of the cells involved, but is indicative of more subtle heritable changes or the potential for their induction. The embryo system discussed here-in provides a unique opportunity to assess the consequences of mutagenic damage. The potential ability to assess the mutagenic, carcinogenic and toxic

impact of chemicals in the same system, with full siblings for al 1 types of study is unrivalled in toxicology, mutagenesis, and carcinogenisis reseach. The efficiency of embryo's for assessing mutagenic/genotoxic damage due to EMS leads to the speculation that this system, which has been used in field investigations (Longwell, 1978; Longwell and Hughes, 1980) and laboratory investigations (this study;Hose et al., 1982,1983) is equally applicable to in-situ exposures and will provide a very sensitive measure of the genotoxic/mutagenic potential of waterborne chemicals.

SUMMARY

1) Brachydanio rerio proved to be a very suitable test species, being capable of consistently producing a large number of embryo's for studies with minor variability.
2) A growth reduction was induced by EMS at sub-lethal concentrations, the gastrula and mid-embryo being the most sensitive periods, based on developmental stage, while growth rate (mitotic index) showed an insignificant reduction at 12 and 24 hours with increasing dose.
3) The analysis of micronuclei indicated an insignificant increase in genetic damage at 24 hours of age in an extra-embryonic tissue.
4) The total frequency of anaphase aberrations proved to be a very sensitive indicator of genetic damage due to EMS. Comparison with published reports indicates that this approach is as sensitive as any yet reported in fish, with numerous advantages over other approaches.
5) The relative sensitivity of the endpoints monitored in this study are as follows:

   Anaphase Aberrations > Micronuclei > Mitotic Index > Developmental Stage > Survival

CONCLUSION

The use of anaphase aberrations in Brachydanio rerio embryo's provides a very sensitive measure of aquatic chemical genotoxicity.

RECOMMENDATIONS

1) Validation of this test approach should continue with other chemical genotoxicants and toxicants to determine it's reliability for detecting a variety of classes of compounds. Further investigation of the micronucleus assay in embryo's is warranted.
2) An investigation of the relationship between the genotoxic damage seen in embryo's and eventual health effects in older fish should be instigated, including reproductive effects, mutagenesis, teratogenesis and carcinogenesis.

ACKNOWLEDGMENTS

The author's wish to thank the Ontario Ministry of the Environment for their support of this project, and the supervisory committee for I.R.S. composed of Drs. J.B. Sprague, H.F. Ferguson, D.R. Rokosh and Mr. G. R. Craig for their aid and encouragement. The help, technical expertise, enthusiasm and space provided by the following persons and groups is gratefully acknowledged: Mr. T. Lovasz, Dr. M. Goldberg, and the Biohazards Laboratory and Toxicity Unit's of the Ministry of the Environment. A special thanks is extended to D. R. Walker for graphical and technical assisstance as well as for critically reviewing this manuscript.

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THE EVALUATION AND APPLICATION OF PULSED NUCLEAR MAGNETIC RESONANCE IN THE ANALYSIS OF ENVIRONMENTAL SAMPLES

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ABSTRACT

THE EVALUATION AND APPLICATION OF PULSED NUCLEAR MAGNETIC RESONANCE IN THE ANALYSIS OF ENVIROMENTAL SAMPLES

The possibility of applying the low resolution pulsed N M R technique to the analysis of enviromental samples is described. The transversal magnetization for solids decays much faster than that of liquids. Comparison of the magnitudes of the total magnetization measured at different times can be used to determine the solid/liquid ratio or the percentage of liquid in a sample. The theoretical basis will be described along with the assessment of some of the microprocessor programs presently available for the analysis for appropriate samples.

1. INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy has, for the past twenty-five years, been an important instrument in the analysis of molecular systems. The literature abounds with references to the application of this technique to such diverse problems as the elucidation of molecular structure, stereochemical assignments, and the study of dynamic systems.

The past fifteen years has seen a dramatic change in the technology of NMR instrumentation and with it has come an even greater diversity of NMR applications. Pulse Fourier transform (PFT) high resolution spectrometers, some with magnetic field strengths approaching 150,000 gauss and interfaced with a high speed computer, have brought the detection of many magnetic isotopes to levels that make their spectra almost as routine as those of ^{1}H. These instrumental improvements have also facilitated the measurement of molecular relaxation times thereby adding another dimension to the understanding of molecular characteristics (1-3).

In the last ten years, a somewhat different technological approach has emerged. Low resolution pulsed NMR has been applied to a number of diverse chemical situations particularly of a quantitative nature. This method has been used, for example, to determine moisture in food products (4), the ratio of bound-to-free water (5), the fat content and solid-to-liquid ratio of chocolate (6), and the oil in peanut seeds (7). These applications-----fast, reliable, accurate, and a minimum of sample preparation-----suggested a potential for pulse NMR in environmental samples, particularly in the analysis of soils for moisture and organic levels and PCB's in lubricating oils.

Therefore, in conjunction with the Ministry of the Environment Laboratories, the following objectives were set out:

1. to assess the application of low resolution pulsed NMR to particular enviromental samples.
2. to derive suitable experimental metholds in terms of:
   - reproducibility
   - reliability
   - ready calibration
   - speed of analysis
   - ease of operation

2. THE NMR EXPERIMENT

When a molecule bearing hydrogen atoms is placed into a magnetic field B_0, the magnetic moments of the ^1H nuclei precess about the direction of B_0 (along the z-axis) at a rate given by their Larmor frequency (Figure 1)

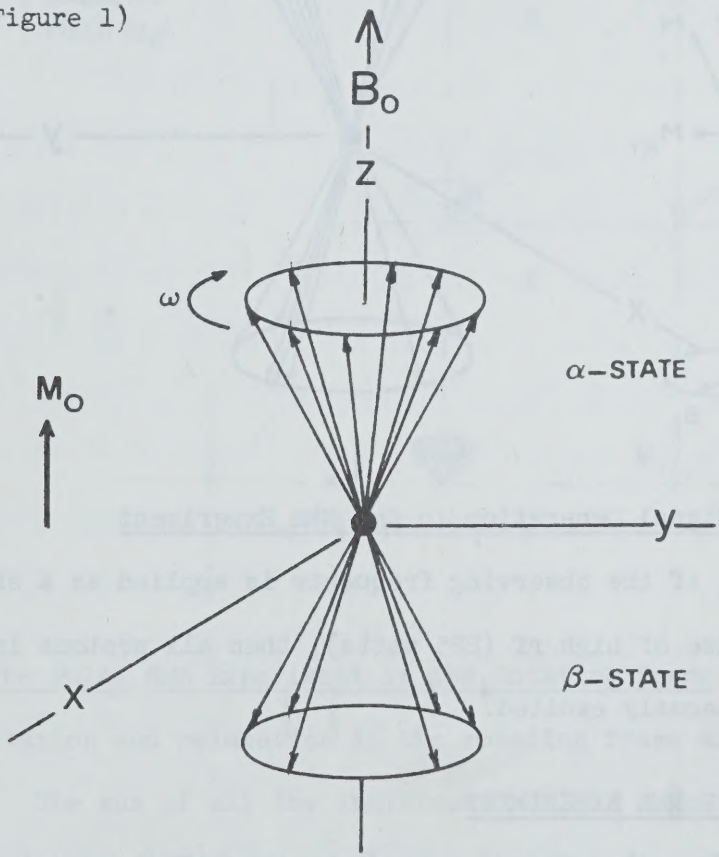

FIGURE 1. The Nucleus in a Magnetic Field

When an observing frequency B_1, applied along the x-axis and polarized in the xy-plane, exactly matches the Larmor frequency of a proton (or group of protons) in the molecule, then a small resultant magnetization in the xy-plane generates a signal (voltage) in the y-axis. (Figure 2)

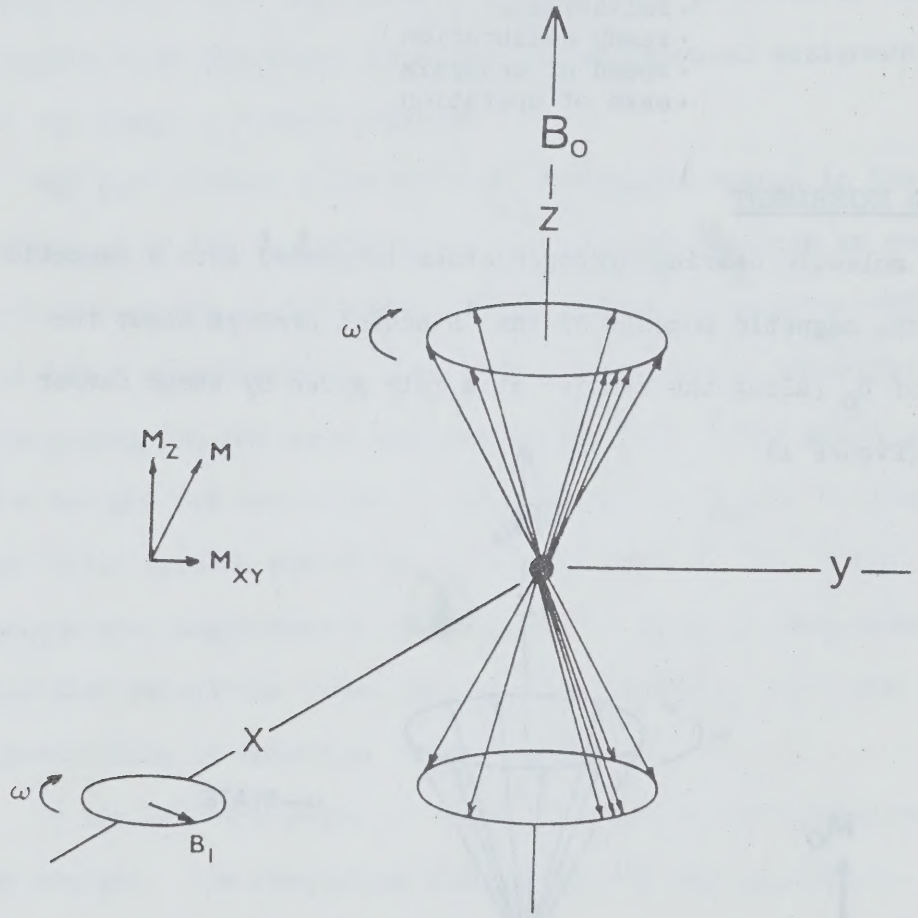

FIGURE 2. Signal Generation in the NMR Experiment

However, if the observing frequency is applied as a short (usec) burst or pulse of high rf (225 watts), then all protons in the sample are simultaneously excited.

3. THE PULSE NMR EXPERIMENT

The pulse nmr experiment is simplified by considering the nuclei in a rotating frame of references. In the rotating

frame of reference. In the rotating frame, the entire coordinate system rotates at the Larmor frequency corresponding to the external magnetic field B_o. The rd field B_o, also oscillating at the Larmor frequency, is therefore fixed in the rotating frame.

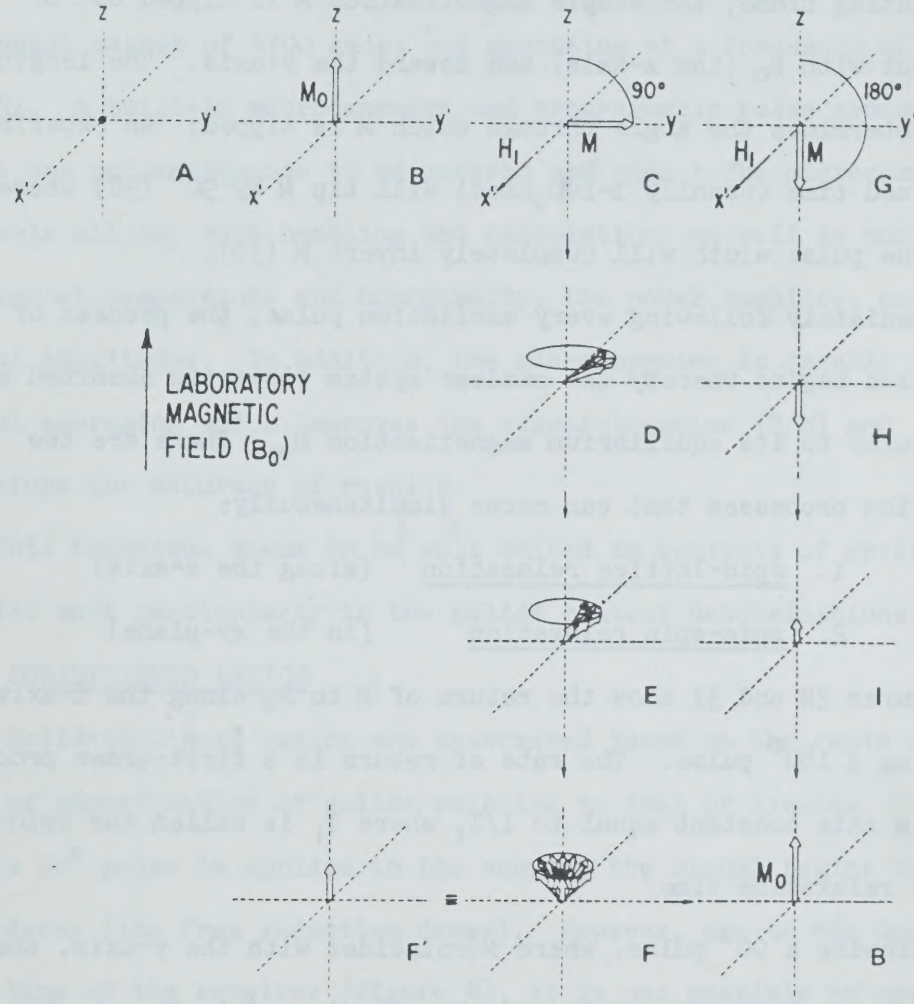

FIGURE 3. The Pulse NMR Experiment in the Rotating Frame

NMR excitation and relaxation in the rotating frame are depicted in Figure 3. The sum of all the individual nuclear magnetic moments is represented by M. When the sample is first introduced into the magnetic field, B_o, no polarization of the nuclear spins is present.

However, the equilibrium magnetization M_o(3B) is quickly established by the interaction of the proton nuclei and the lattice thereby giving an excess of nuclei in the lower energy state according to the Boltzmann distribution law.

When the sample is irradiated by an rf field along the x-axis in the rotating frame, the sample magnetization M is tipped out of alignment with B_o (the z-axis) and toward the y-axis. The length of pulse determines the angle through which M is tipped; an experimentally determined time (usually 1-100 μsec) will tip M by 90^o (3C) whereas twice the pulse width will completely invert M (3G).

Immediately following every excitation pulse, the process of relaxation begins whereby the nuclear system loses its absorbed energy and returns to its equilibrium magnetization M_o. There are two relaxation processes that can occur simultaneously:

1. spin-lattice relaxation (along the z-axis)
2. spin-spin relaxation (in the xy-plane)

Figures 3H and 3I show the return of M to M_o along the z-axis following a 180^o pulse. The rate of return is a first-order process having a rate constant equal to $1/T_1$ where T_1 is called the spin-lattice relaxation time.

Following a 90^o pulse, where M coincides with the y-axis, the signal decays both the z-axis and the xy-axis and in the xy-plane. The latter decay phenomenon, as a function of time, is the free induction decay (FID) from which a normal frequency-domain spectrum can be derived. The decay of the xy magnetization is essentially a dephasing of the magnetic moments and the decay rate is first-order with a rate constant of T_2, the spin-spin relaxation time.

Free induction decay signals decay normally as a function of T_2^* where the latter relaxation time includes contribution to dephasing from the inhomogeneity of the magnetic field.

4. THE PULSED NMR INSTRUMENT AND OPERATION

The Bruker Minispec PC 20 is pulsed NMR instrument having a permanent magnet of 4700 gauss and operating at a frequency of 20 MHz. A built-in microcomputer and programmable pulse generator allow any pulse sequence to be entered and used. The microcomputer controls all the data handling and calculations as well as monitoring the magnet temperature and homogeneity, the power supplies, and the signal amplitudes. In addition, the microcomputer is capable of signal averaging which improves the signal-to-noise (S/N) and therefore the accuracy of results.

This technique seems to be well suited to analysis of environmental samples most particularly in the solids content determinations.

a) SOLID/LIQUID RATIOS

Solid-to-liquid ratios are determined based on the rapid decay of magnetization of solids relative to that of liquids. When a 90° pulse is applied to the sample, the signal begins to decay (the free induction decay). However, due to the dead time of the receiver (Figure 4), it is not possible to measure the initial signal magnitude (s+1), but only (s'+1) which is the signal magnitude a certain time after the 90° pulse. However, a correction factor f can be determined experimentally so that the direct ratio of solid-to-liquid can be calculated (8)

$$\% \text{ solid} = \frac{fs' \times 100}{fs' + 1}$$

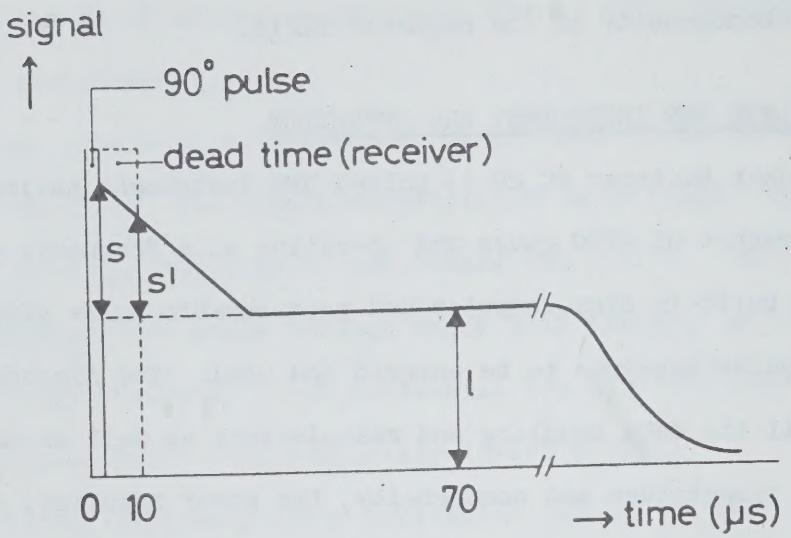

FIGURE 4. Magnetization Decay of a Solid/Liquid Sample

This operation is fully automatic and is begun as soon as the sample tube is inserted into the instrument. The signal is

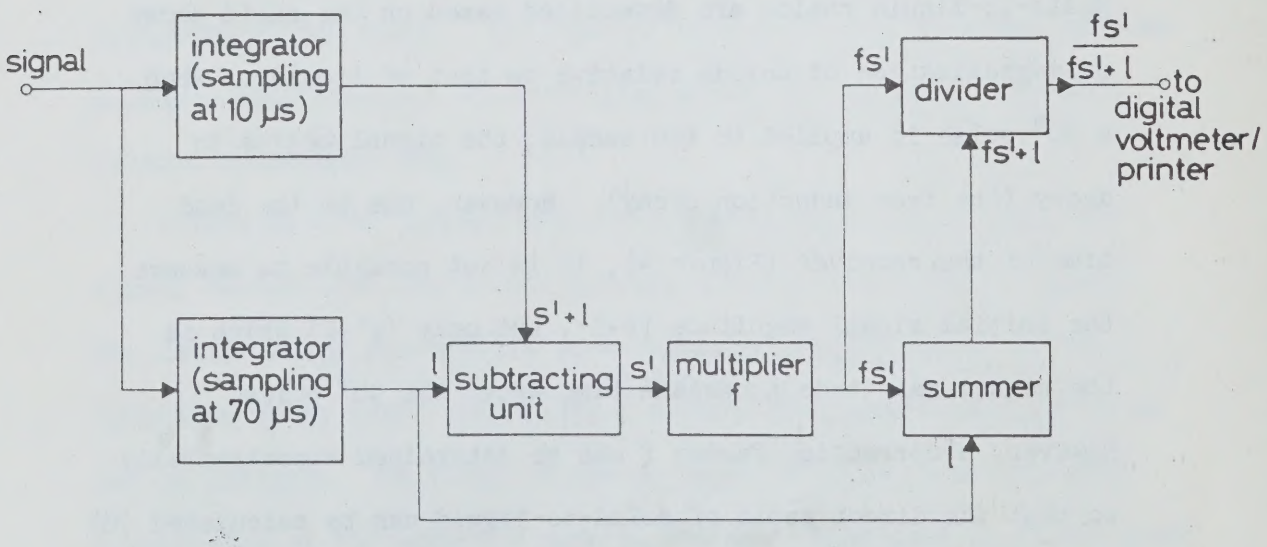

FIGURE 5. Data Processing Operation

sampled at 10 μs and at 70 μs and the respective amplitudes fed into the data processing unit (Figure 5) of the microcomputer (9). The calculated S/L ratio is then printed out and displayed on a digital voltmeter.

b) PROTON QUANTITIES IN SOLIDS OR LIQUIDS

By shifting the point of signal measurement, the proton quantities can be determined. As shown in Figure 4, signal measurement at 70 μs will be due only to the liquid component. A calibration curve (Figure 6) can be constructed by using standard samples. All the information, including a least squares fit of the data, is stored in the microcomputer so that when a sample of unknown concentration is inserted into the instrument, the concentration is rapidly determined are printed out.

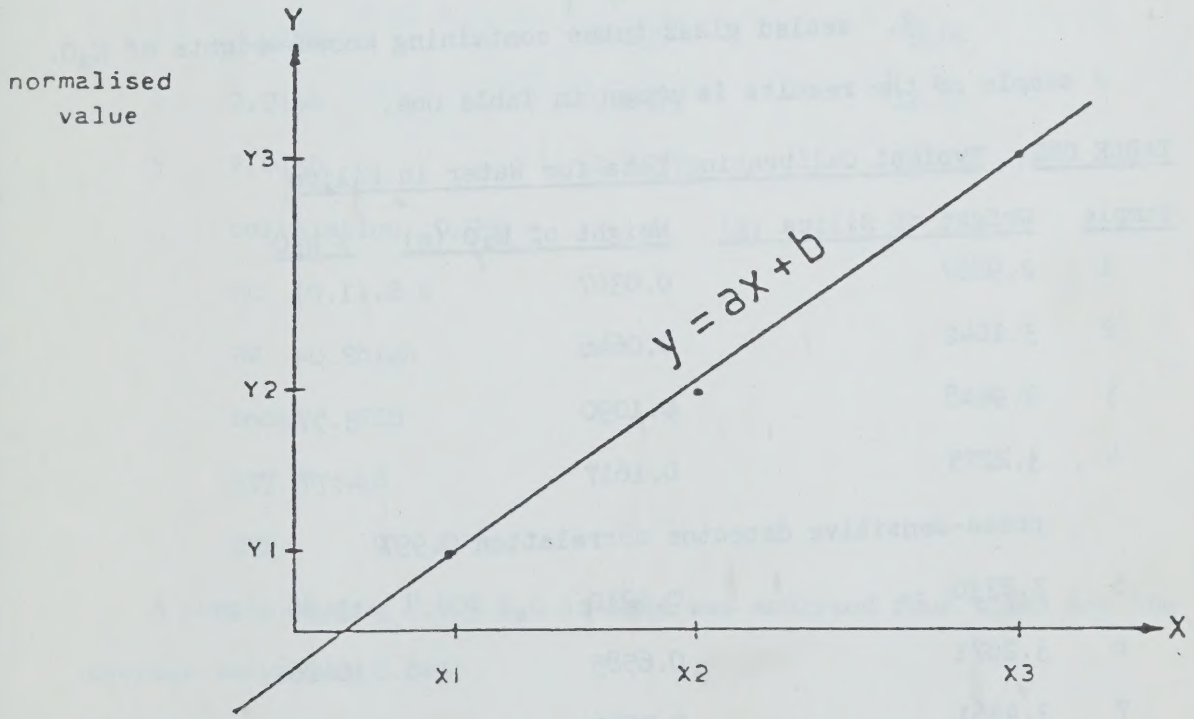

FIGURE 6. Normalization Calibration Curve

5. EXPERIMENTAL AND RESULTS

The Bruker PC 20 Multispec NMR was delivered in May 1983. Initial objectives were as follows:

(i) to learn the operation of the instrument and to determine methods of calibration.

(ii) to analyze samples of known concentration in the assessment of various microcomputer programs that are available.

(iii) to determine the instrument parameters (number of scans, delay time, attenuation, initial delays, etc.) appropriate for a given analysis.

a) CALIBRATION

Standard samples of water in silica were prepared and analyzed.

Three methods of sample preparation were utilized:

1. individual samples having a known % H_2O
2. standard addition method from a single individual sample
3. sealed glass tubes containing known weights of H_2O.

A sample of the results is shown in Table One.

TABLE ONE. Typical Calibration Data for Water in Silica

Sample	Weight of Silica (g)	Weight of H_2O (g)	% H_2O
1	2.9887	0.0307	1.01
2	3.1042	0.0646	2.04
3	2.9448	0.1090	3.57
4	3.2275	0.1617	4.77
	phase-sensitive detector correlation 0.997		
5	3.2330	0.5212	13.88
6	3.2671	0.6585	16.86
7	3.4361	0.7557	18.03
8	3.3421	1.0917	24.62
	diode detector correlation 0.999		

While all three preparation methods gave essentially the same results, it was evident that the use of sealed calibration tubes negated any interactions likely to occur in natural samples. It was concluded that the more conventional sample preparation methods would be used in subsequent work. In addition there was no appreciable difference in these results when the 90° pulse or the 90°-τ-180° pulse sequence were used.

A typical moisture analysis is recorded in Table Two. Although instrument calibration may take 15-30 minutes, the analysis of an individual unknown is complete in only a few minutes.

TABLE TWO. Analysis for Moisture in Sand

Sample	Mass of Sand (g)	Mass of H_2O (g)	% H_2O
1	2.0157	0.0000	0.00
2	2.0129	0.0995	4.71
3	2.0160	0.1994	9.00
4	2.0194	0.2977	12.85
5	2.0130	0.3957	16.43

correlation 0.993

RD 1.2 S

BW high

mode PSD

ATT 18

ENH 9

A sample having 8.60% H_2O in sand was analyzed four times and the average value was 8.84%.

b) SOIL/WATER

Soil samples were dried at 105°C and calibration samples prepared. Attempts to calibrate the instrument proved futile

since the presence of paramagnetic materials in the roil reduced considerably the relaxation time of the water.

Recent experiments are focussing on the spin-echo pulse sequence (90^{o}-τ-180^{o}) which should eliminate this limitation.

ACKNOWLEDGEMENTS

The authors are grateful to the Ontario Ministry of the Environment for financial supports.

In particular, our appreciation is extended to Dr. M. Moselhy and Dr. O. Meresz for their help and useful discussions.

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Aquatic Toxicity Studies of Multiple Organic Compounds
The Concentration Addition Model

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Ontario Ministry of the Environment

Toronto, November, 1983

Abstract

A discussion of the various models proposed by aquatic toxicologists for predicting the joint toxicity of mixtures of organic compounds from their individual toxicities is presented. The rationale for adopting the "concentration addition" model as the most relevent to acute toxicity in fish is demonstrated by mixture data generated by our research group on chlorinated benzenes and phenols.

Whether or not concentration addition can successfully predict chronic effects such as fish growth presently remains uncertain because of an inadequate data base for chlorinated benzenes and phenols.

Bioconcentration data on chlorinated benzene mixtures clearly demonstrates that this group of organics bioconcentrate in fish independently of each other, which is consistent with concentration additivity. However, independent bioconcentration is only successful as a predictive model when BCFs are compared on a lipid weight basis.

Introduction

Water quality criteria have traditionally been based on no-effect levels of single toxicants. But the real aquatic environment experiences a continuous bombardment of many toxic substances. Effluents from industrial operations and sewage treatment plants (STPs), along with atmospheric fallout, always contain several pollutants of an organic and/or inorganic nature. Many of these toxicants, particularly the organics referred to as "priority pollutants" (Keith and Telliard, 1979) have been studied extensively in terms of their individual toxicity to aquatic organisms and their potential to concentrate in biological systems. Surprisingly, the joint action of mixtures of organic toxicants is a research area which has received limited attention from aquatic toxicologists. In addition, those responsible for establishing water quality standards have rarely addressed the real issue of multiple organic contamination of aquatic ecosystems. Therefore, the purpose of the investigation by the Aquatic Toxicity Research Group at Lakehead University is to experimentally validate the most appropriate model (or models) proposed by aquatic toxicologists for predicting the joint toxicity of organic mixtures at both the acute and chronic levels. A further objective is to determine whether or not any of the proposed models are useful in predicting bioconcentration of multiple organic compounds.

A brief discussion of the types of joint action arising from mixtures of toxicants is necessary to appreciate the method of approach taken in this study.

A classification by Bliss (1939), which was later expanded by Plackett and Hewlett (1952), describes four types of joint action: 1) simple similar; 2) independent; 3) complex similar; and, 4) dependent (Fig. 1).

A toxicant causes a response by producing changes in a physiological system referred to as the site of action. Similar joint action occurs when two toxicants act at a common site. Conversely, dissimilar joint action arises when the toxicants act at different sites. Such joint action can be interactive if one toxicant influences the biological action of the other or, non-interactive if neither toxicant influences the effect of the other. Mathematical expressions for quantifying joint actions with this system of classification can be readily developed for binary mixtures. However, when more than two chemicals are present, joint action of different pairs can fall into different classes because additional joint actions are possible between the pairs. Therefore, a quantitative description for mixtures of more than two components is not possible when interaction is present; that is, when the joint action is complex similar or dependent.

In a recent publication by Konemann (1981), a Mixture Toxicity Index (MTI) was proposed for comparing quantitatively the results of acute multiple component toxicity tests (Fig. 2). The main advantage of Konemann's mixture toxicity scale over other mathematical expressions such as the Additivity Index proposed by Marking (1977) is that constant values are obtained for two reference points, "concentration addition" and "no addition", independently of the number of compounds and the ratio between the concentrations. These reference points require

Fig. 01. Classification of joint toxic actions (modified from Plackett and Hewlett, 1952).

	Similar joint action	Dissimilar joint action
Non-interaction	simple similar	independent
Interaction	complex similar	dependent

Fig. 02. The Mixture Toxicity Index (MTI) - a mixture toxicity scale (modified from Konemann, 1981).

MTI	Classification of possible types of joint action
MTI < 0	Antagonism
MTI = 0	No addition (independent, r = +1)
0 < MTI < 1	Partial addition
MTI = 1	Concentration addition (simple similar)
MTI > 1	Supra-addition (potentiation or synergism)

further discussion.

The mathematical model for simple similar action is the "toxic unit" concept (Sprague, 1970). Each component of a mixture can be substituted by an equitoxic concentration of an other chemical with simple similar action without altering the reponse. This procedure is called concentration addition by Anderson and Weber (1975).

The no addition reference point is the case of independent action. It occurs when the components of a mixture affect different sites of action but the ultimate reponse is the same - mortality. Anderson and Weber refer to the procedure for calculating the response to this type of joint action as response addition. Independent action can be readily calculated when there is a positive correlation ($r = +1$) between susceptabilities of the individual test organisms to the components of the mixture. This relationship, called the "tolerance correlation" by Konemann, cannot be negative for multiple component mixtures and response addition with $r = 0$ (no tolerance correlation) cannot be used to predict the toxicity of such mixtures with reasonable accuracy (Konemann, 1981).

The MTI, therefore, offers us two mathematical models which have fixed values and for which toxicity experiments can be designed to quantitatively evaluate the joint toxicity of mixtures.

Our approach to the toxicity of multiple organic compounds has been based on the toxic unit concept. The rationale for selecting concentration over response addition as the most useful model for predicting joint toxicity was supported by two

criteria:1) historically, pharmacologists and other scientists believe that independent action (response addition) is less likely to occur in reality over other forms of joint toxicity because an organism is at least largely a coordinated whole (Plackett and Hewlett, 1967; Konemann, 1981); and, 2) the nature of the chemicals selected for study - the chlorinated benzenes (CBs) and the chlorinated phenols (CPs). The second criterium is perhaps the most important.

The CBs and CPs which we are studying are illustrated in Figs. 3 and 4. They are first and foremost organic compounds and, despite their structural differences, they do have a common denominator. They are all derivatives of the benzene ring structure and therefore, are more or less lipophilic chemicals. Lipophilic (or hydrophobic) compounds can have a specific mode of toxic action (i.e. primary site of action). For example, there is evidence that PCP interferes with mitochondrial oxidative phosphorylation (Weinbach and Garbus, 1965). However, lipophilic compounds also induce narcotic effects in mammals and fish - membrane perturbation, leading to anesthesia and progressive central nervous system depressions - which are chemically nonspecific (Hermens and Leeuwangh, 1982; Veith et al., 1983). The CBs are excellent examples of narcotic chemicals with a secondary (nonspecific) site of action. But every lipophilic compound can exert this narcotic effect even though it may not be apparent with chemicals like PCP due to the overwhelming effect at the primary (specific) site of action.

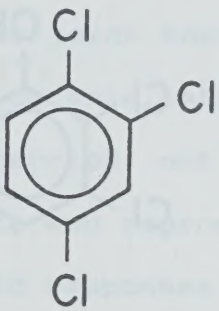
We think the joint toxicity of CB and CP mixtures is best

Fig. 03. Chlorinated benzene compounds studied.

1,4 -dichlorobenzene

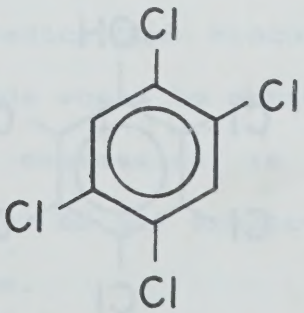
1,4 - DCB
m.w. = 147.01
m.p. = 53 C

1,2,4 - trichlorobenzene



1,2,4 - TCB
m.w. = 181.46
m.p. = 17 C

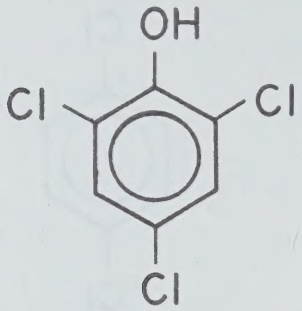
1,2,4,5 - tetrachlorobenzene



1,2,4,5 - TTCB
m.w. = 215.90
m.p. = 139 C

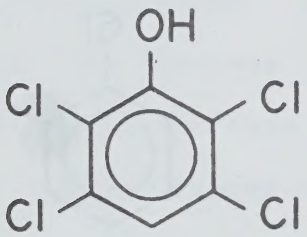
Fig. 04. Chlorinated phenol compounds studied.

2,4,6 - trichlorophenol



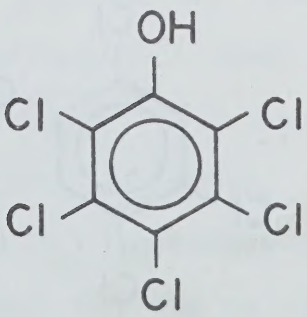
2,4,6 - TCP
m.w. = 197.46
m.p. = 68 C

2,3,5,6 - tetrachlorophenol



2,3,5,6 - TTCP
m.w. = 231.91
m.p. = 115 C

pentachlorophenol



PCP
m.w. = 266.35
m.p. = 191 C

characterized by simple similar action due to the narcotic effect exhibited by these compounds. The most relevent model, therefore, is Konemann's equation for concentration addition (Fig. 5). To be sure, interaction would result in complex similar and/or dependent joint actions, but we have no mathematical model to quantitatively predict these types of joint toxicity.

So far, we have only discussed joint toxicity in terms of acute response (i.e. mortality). Is concentration addition for CB and CP mixtures a relevent model for predicting chronic effects such as fish growth or is response addition more appropriate for sublethal parameters? Very little information is available in the world's literature on chronic joint toxicity. Alabaster (1981) has stated that the few chronic studies with mixtures of toxicants that have been carried out indicate a less-than-additive effect on fish growth and reproduction. However, there is no evidence that chronic responses are non-additive (i.e. independent). A logical approach, therefore, was to extend the toxic unit concept into the design of our Early Life Stage (ELS) toxicity tests to confirm Alabaster's report that chronic joint toxicity demonstrates partial addition.

To be able to predict the bioconcentration dynamics of multiple organic compounds would be extremely useful information. Again, a mathematical expression is necessary in order to evaluate the usefulness of any predictive model for multiple compound bioconcentration.

Our approach towards this aspect of the project was to determine if any of the existing joint toxicity models might be

Fig. 05. The mathematical expression for concentration addition or simple similar action (modified from Konemann, 1981).

$$\sum_{i=1}^{i=n} f_i = 1 \text{ a.t.u.}$$

where: f_i = water concentration of chemical, i

LC_{50} of i

n = number of components in the mixture

a.t.u. = acute toxic units

Fig. 06. Calculation of the MTI value for equitoxic mixtures (modified from Konemann, 1981; Hermens and Leeuwangh, 1982).

$$MTI = 1 - \frac{\log M}{\log n}$$

where: M = total a.t.u.

n = number of components in the mixture

appropriate for predicting joint bioconcentration. The Structure-Activity Group at the EPA laboratory in Duluth have found in a limited number of tests that organic compounds bioconcentrate in fish independently of each other (Broderius, 1981). The term "independent" in this context, however, should not be interpreted as being synonymous with response addition, discussed under joint toxicity. Non-interactive is perhaps a better descripter than independent in this context. Furthermore, it is well known that organic compounds bioconcentrate in a target tissue - the body lipids. Hence, the lipid tissue can be thought of as a common "site of action". Because interaction is not present, it seems feasible that at least the concept of concentration addition may be appropriate for predicting joint bioconcentration of organic chemicals. Indeed, the mathematical expression for simple similar action , presented in Fig. 5, may even be adaptable for quantifying the process.

With this outline of the objectives and detailed discussion of the approach taken in this study, let us now take a look at the practical application of the concentration addition model for joint toxicity and bioconcentration.

Protocol

Details of the experimental protocol for the toxicity and bioconcentration tests performed during the study was presented last year at this conference (Ozburn *et al.*, 1982). A brief reiteration of the tests may be helpful at this point before reviewing the data generated to date.

1. Acute Testing

Ninety-six hour exposures with flagfish were used to generate LC_{50} values for individual chemicals and for multiple component mixtures. The acute tests employed both static and flow-through techniques. The data reported for the static "rangefinder" tests are based on nominal (non-measured) chemical concentrations, whereas the data generated by flow-through methods utilized measured concentrations determined by gas chromatographic analyses. The LC_{50}s for individual chemicals were calculated using the familiar mg/l (ppm) format. For the mixture tests, mg/l were converted to "acute toxic units" (a.t.u.) according to Konemann's formula for simple similar action (Fig. 5).In order to compare the results of our acute mixture tests, MTI values were also calculated using Konemann's formula for equitoxic mixtures (Fig. 6), and the joint action classified according to his mixture toxicity scale presented in Fig. 2.

2. Chronic Testing

Our ELS toxicity test utilizes two age groups of flagfish in simultaneous flow-through exposures: 1) embryo/larval fish, with data collected on hatching success and 10-day survival; and, 2) one week fry, with data generated on survival and growth (weight) over 28 days.

MATCs are then determined for individual chemicals. The MATC (maximum acceptable toxicant concentration) is defined as the hypothetical toxic threshold concentration falling between the highest concentration showing no effect and the next highest toxicant concentration showing a significant toxic effect (Mount

and Stephen, 1967). The hypothetical toxic threshold concentration is called the "chronic value" and is quantified as the geometric mean of the no effect/effect limits. A chronic value is calculated for each response parameter (e.g. hatchability, growth, etc.).

The chronic values determined on individual chemicals are expressed in ug/l (ppb). They are used to convert the concentrations of the mixture's components (also measured in ug/l) to "chronic toxic units" (c.t.u.). by applying essentially the same concentration addition formula as that presented in Fig. 5. In this case, however, f_i is the ratio of the water concentration of component i, to the chronic value of component i. Hence, c.t.u. can be calculated for any chronic response in a multiple component test provided chronic values are obtained for that response in the tests conducted with individual compounds.

3. Bioconcentration Testing

Our flagfish bioconcentration test consists of a 28-day exposure to 5 ug/l of a chemical (the uptake phase) followed by a 14-day depuration period in clean water (the clearance phase). Fish are sampled for total chemical body burden at predetermined intervals during both uptake and clearance.
The bioconcentration data is expressed not only on the basis of total body weight, but also on lipid (weight) - the target tissue or, "site of action".

The only multiple component bioconcentration test that has been conducted so far is a CB mixture. In this presentation we will attempt to apply the principle of concentration addition to

the data. We will not attempt, at this time, to develop a quantitative expression for joint bioconcentration until the mixture work with the CPs is completed.

With this brief review of the tests, we can now discuss the data.

Results and Discussion

1. Acute Testing

The non-measured, static tests performed on individual CBs yielded LC_{50} values for flagfish similar to those reported for bluegill under comparable test conditions; the expected trend of increasing toxicity with degree of chlorination is readily apparent in the data (Table 1).

When the flagfish LC_{50} values from Table 1 were used in a triple component CB static to establish a nominal concentration gradient based on Konemann's formula (Fig. 5), an LC_{50} of 1.41 a.t.u. was generated instead of the expected 1.0 value (Table 2). However, a non-measured static is only a "rangefinder" test and has limited precision. Furthermore, the nominal concentration gradient may be too wide to provide a reasonable estimate of the LC_{50} and associated confidence limits, particularly with a data set which lacks partial mortalities (i.e. no per cent dead between 0 and 100).

The single compound, flow-through LC_{50}s generated for 1,4-DCB and 1,2,4-TCB are consistent with the trend of increasing

Table 01. Nominal 96-hour LC_{50}s for 1,4- dichlorobenzene, 1,2,4-trichlorobenzene and 1,2,4,5- tetrachlorobenzene to juvenile flagfish. [a]

CB compound	Trimmed Spearman - Karber Analyses LC_{50}, mg/l flagfish	bluegill [b]
1,4 - DCB	4.481 (4.022-4.993) [c]	4.28
1,2,4 - TCB	4.000 (3.591-4.454)	3.36
1,2,4,5 - TTCB	2.078 (1.470-2.939)	1.55

[a] non-measured static tests with 24-hour solution renewal and acetone carrier at 196 mg/l.

[b] U.S. EPA, 1978.

[c] 95 % confidence limits.

Table 02. Nominal 96-hour LC_{50} for a triple CB mixture to juvenile flagfish. [a]

Test level	Concentration, [b] acute toxic units	Number exposed	Number dead
0	0	10	0
1	0.25	10	0
2	0.5	10	0
3	1.0	10	0
4	2.0	10	10
5	4.0	10	10

Trimmed Spearman - Karber Analysis

LC_{50}	95 % confidence limits	% trim
1.414	(unreliable)	0

a non-measured test with 24-hour solution renewal and acetone carrier at 196 mg/l.

b acute toxic units calculated from the concentration addition formula (Fig. 5).

toxicity with degree of chlorination; the apparent anomaly to this trend is 1,2,4,5-TTCB (Table 3). We expected this compound to have the smallest LC_{50}, not the largest.

The TTCB isomer does not fit the expected toxicity pattern because of its poor solubility in water. The solubility of 1,2,4,5 - TTCB at 25 C is ~ 0.5 mg/l (A.T.R.G., 1982).However, concentrations of the chemical measured within the solubility range during the 96-hour acute test produced no mortality (Table 4). Furthermore, follow-up testing with saturated solutions of the compound produced no flagfish mortality after 10 days of exposure, even though toxic signs (narcotic effects) were evident. We concluded that 1,2,4,5 - TTCB is not acutely toxic within 96 hours at water saturation.

But exposure tanks dosed at concentrations above the compound's solubility (test levels 3 to 5) does yield an acute response (Table 4). A detailed discussion of the physico-chemical behaviour of 1,2,4,5 - TTCB was presented at last year's conference (Ozburn et al., 1982) and will not be repeated here. The important point to remember, however, is that the observed toxicity of 1,2,4,5 - TTCB (i.e. the LC_{50} reported in Tables 3 and 4) is a function of both the dissolved portion of the chemical plus the particle size and distribution of the insoluble component.

Using the LC_{50} values from Table 3 in a measured, flow-through CB triple mixture test yields an LC_{50} of 1.4 a.t.u. (Table 5) - the same estimate obtained under static test conditions. It appears that the experimental data does not fit

Table 03. Measured 96-hour LC_{50}s for 1,4- dichlorobenzene, 1,2,4-trichlorobenzene and 1,2,4,5- tetrachlorobenzene to juvenile flagfish. [a]

CB compound	Concentration, mg/l LC_{50}	95 % confidence limits	% trim
1,4 - DCB	2.053	1.955-2.156	0
1,2,4 - TCB	1.217	1.081-1.371	0
1,2,4,5, - TTCB	2.150	1.853-2.495	35

[a] flow-through tests with acetone carrier at 196 mg/l.

Table 04. Acute toxicity of 1,2,4,5 - tetrachlorobenzene to juvenile flagfish. [a]

Test level	Concentration, [b] mg/l	Number exposed	Number dead
0	0	20	0
1	0.148±0.009 [c]	20	0
2	0.373±0.055	20	0
3	0.695±0.050	20	4
4	1.855±0.326	20	7
5	2.493±0.357	20	13

Trimmed Spearman - Karber Analysis

LC_{50}	95 % confidence limits	% trim
2.150	1.853-2.495	35

[a] flow-through 96-hour test with acetone carrier at 196 mg/l.

[b] glass sintered samples.

[c] mean ± s.d.

Table 05. Acute toxicity of a triple CB mixture to juvenile flagfish. [a]

Test level	Concentration, acute toxic units [b] 1,4-DCB a.t.u.	1,2,4-TCB a.t.u.	1,2,4,5-TTCB a.t.u.	Total a.t.u.	Number exposed	Number dead
0	0	0	0	0	40	0
1	0.211	0.220	0.172	0.604	40	0
2	0.194	0.237	0.224	0.655	30	1
3	0.230	0.276	0.255	0.761	10	0
4	0.457	0.390	0.159	1.006	10	3
5	0.371	0.440	0.382	1.193	30	1
6	0.434	0.502	0.413	1.348	10	5
7	0.733	0.683	0.112	1.528	10	10
8	0.501	0.583	0.609	1.693	10	2
9	0.882	0.814	0.155	1.851	10	10

Trimmed Spearman - Karber Analysis

LC_{50}	95 % confidence limits	% trim	MTI value
1.402	1.296 - 1.517	0	0.69

[a] flow-through 96-hour test with acetone carrier at 196 mg/l.

[b] acute toxic units calculated from the concentration addition formula (Fig. 5).

the concentration addition model. Applying Konemann's equation from Fig. 6, the calculated MTI value is 0.69 and the joint action would be classified as partial addition: 0< MTI < 1 (Fig. 2).

In experiments with guppies, Konemann (1981) also found that a mixture of DCB, TCB and TTCB generated an LC_{50} estimate of 1.4 a.t.u. when the mixture's toxic strength was calculated from experimental LC_{50}s determined on individual components. But when he calculated the individual LC_{50}s on the basis of their QSARs (Quantitative Structure Activity Relationships) he discovered that the mixture's toxic strength was exactly equal to 1 a.t.u. and hence, fit the concentration addition model for simple similar action. His explanation for the discrepancy in the experimental data was that the CBs do not have the same primary site of action. They do have at least one common site of action which is represented by the QSAR calculations, but that common site of action is primary for some CBs and secondary for others.

Our explanation as to why the experimental data does not fit the proposed model is based on TTCB's lack of acute toxicity at water solubility.

The relative contribution of 1,2,4,5 - TTCB to total toxicity at every test level was very small compared to the contributions of the DCB and TCB components. In fact, at test level 8 where the TTCB component was at its maximum contribution to the total a.t.u. value, an actual decrease in mortality was observed (Table 5). We think TTCB was contributing little or nothing to the mixture's total toxic strength.

If we remove the TTCB component and reanalyze the triple CB mixture data using only the DCB and TCB components to calculate the concentration gradient, we generate an LC_{50} of 1.07 a.t.u.; the equivalent MTI value is 0.90 (Table 6). The same data set utilizing the upper 95 % confidence limit of the individual LC_{50}s for 1,4, - DCB and 1,2,4 - TCB (from Table 3) will yield an LC_{50} value for the test of 0.99 a.t.u.

It appears that DCB and TCB do exhibit simple similar action while TTCB does not. TTCB may or may not have the same common site of action as suggested by Konemann, but we know that it is non- toxic at water solubility (within 10 days) and therefore concentration addition is not demonstrated when the compound's LC_{50} is based primarily on suspended particles of unknown toxicity and unknown mode of action.

We did attempt to follow-up the CB triple mixture test with a flow-through DCB/TCB binary test. However, analytical problems occurred with the water analyses during the actual test, which were not discovered until after the test was completed. Hence, the only reliable water chemistry available was a single preliminary analysis performed after the flow-through system was stabilized but two days prior to introducing the test fish. Although this test cannot be regarded as a valid demonstration of DCB/TCB concentration addition, we did take the trouble to "crunch the numbers". Using the upper 95 % confidence limit of the LC_{50} estimates for 1,4 - DCB and 1,2,4 - TCB from Table 3, the biological response, when fitted to the single chemistry analysis, yields a 95 % confidence limit for the LC_{50} of 1.024 to 1.312 a.t.u. The MTI value for 1.024 a.t.u. is 0.97.

Table 06. Acute toxicity of a triple CB mixture WITHOUT the TTCB component to juvenile flagfish. [a]

Test level	Concentration, acute toxic units [b]			Number exposed	Number dead
	Total	- 1,2,4,5-TTCB	= (1,4-DCB+1,2,4-TCB)		
	a.t.u.	a.t.u.	a.t.u.		
0	0	0	0	40	0
1	0.604	0.172	0.432	40	0
2	0.655	0.224	0.431	30	1
3	0.761	0.255	0.506	10	0
4	1.006	0.159	0.847	10	3
5	1.193	0.382	0.811	30	1
6	1.348	0.413	0.935	10	5
7	1.528	0.112	1.416	10	10
8	1.693	0.609	1.084	10	2
9	1.851	0.155	1.696	10	10

Trimmed Spearman - Karber Analysis

LC_{50}	95 % confidence limits	% trim	MTI value
1.074	0.990 - 1.165	1.25	0.90

[a] flow-through 96-hour test with acetone carrier at 196 mg/l.

[b] acute toxic units calculated from the concentration addition formula (Fig. 5).

We think our acute data on CB mixtures supports the concentration addition model of joint toxic action for this group of chemicals.

Results of the non-measured, static tests performed on individual CPs are presented in Table 7. Again, the anticipated trend of increasing toxicity with degree of chlorination is readily demonstrated by the data. Our results, however, are significantly different from those reported for bluegill under static test conditions (Table 7). It's true that non-measured statics do not give particularly accurate LC_{50} estimates, but the large discrepancy between the flagfish and bluegill data cannot be explained solely on the basis of the inadequacies of the static test method. Sensitivity of the species to the toxicant is also not likely to account for these differences.

We think the differences are probably due to two characteristics of the CP compounds: 1) chemical purity; and, 2) effect of water pH on toxicity.

CP compounds can contain varying amounts of impurities, which is usually a result of process chemistry (Jones,1981). The moat prominant of these impurities are the polychlorinated dibenzo-p-dioxins , (PCDDs) , and also the polychlorinated dibenzofurans (PCDFs), which are highly toxic compounds in themselves. Commercial grade CPs can vary considerably with repect to the quantities of PCDDs and PCDFs in their composition. Therfore, the chemical purity of a CP compound can result in big differences in reported LC_{50} estimates.

The other important characteristic of CPs is that their

Table 07. Nominal 96-hour LC_{50}s for 2,4,6- trichlorophenol, 2,3,5,6- tetrachlorophenol and pentachlorophenol to juvenile flagfish. [a]

CP compound	Trimmed Spearman - Karber Analyses LC_{50}, mg/l	
	flagfish	bluegill [b]
2,4,6, - TCP	2.264 (2.021-2.537) [c]	0.320
2,3,5,6 -TTCP	0.564 (0.458-0.694)	0.170
PCP	0.186 (0.119-0.292)	0.020-0.060

[a] non-measured static tests with 24-hour solution renewal and acetone carrier at 79 mg/l.

[b] Ontario M.O.E., 1983.

[c] 95 % confidence limits.

toxicity decreases with increasing water pH (Holcombe et al., 1980). The CPs dissociate in water to chlorophenate ions, which are less toxic than the undissociated form. The extent of dissociation increases as pH increases and thus, the toxicity of the compound decreases. Hence, the pH of the diluent water in the flagfish and bluegill static tests reported in Table 7 may account for the observed differences in acute toxicity.

In our study, we have used the purest grades of CPs available, in order to reduce joint effects from highly toxic impurities. As an added precaution, the CPs were purified further in our laboratory by a variety of techniques.

The pH (in the laboratory) of our Lake Superior water supply is ~ 7 (Ozburn et al., 1982).Thus, our near-neutral diluent water does not contribute significantly to dissociation of the CP chemicals under study. The degree of dissociation is almost entirely based on the dissociation constant of the specific CP isomers.

When the flagfish nominal LC_{50} values from Table 7 were used in a triple component CP static test, an LC_{50} of 0.53 a.t.u. was obtained (Table 8). With a result of only half a toxic unit, the data suggests that the supra-addition model would be a better descripter of the joint toxic action of these CP compounds.Indeed, the calculated MTI value for the test is 1.58. However, we proceeded with the concentration addition concept because the single compound, flow-through LC_{50} generated for 2,3,5,6 - TTCP was significantly different from that obtained in the static test. The flow-through LC_{50}s for 2,4,6, - TCP and PCP

Table 08. Nominal 96-hour LC_{50} for a triple CP mixture to juvenile flagfish. [a]

Test level	Concentration, [b] acute toxic units	Number exposed	Number dead
0	0	10	0
1	0.175	10	0
2	0.351	10	0
3	0.702	10	9
4	1.404	10	10
5	2.807	10	10
6	5.614	10	10

Trimmed Spearman -Karber Analysis

LC_{50}	95 % confidence limits	% trim
0.532	0.466-0.607	0

a non-measured test with 24-hour solution renewal and acetone carrier at 79 mg/l.

b acute toxic units calculated from the concentration addition formula (Fig. 5).

were consistent with the static estimates but 2,3,5,6 - TTCP gave an LC_{50} estimate two-fold larger (Table 9). Now we needed an explanation as to why the static and flow-through techniques yielded such different results for the TTCP compound.

Although both test methods gave numbers which were consistent with the trend of increasing toxicity with increasing chlorination, the flow-through LC_{50} of 1.16 mg/l for TTCP appeared to be a more reasonable estimate of its toxicity in relation to TCP and PCP. Besides, we tend to accept a measured flow-through estimate as a more valid number.

Because we decided that the static LC_{50} for TTCP was wrong (particularly when the nominal CP mixture test gave a result no where near 1 a.t.u.), we proceeded with a triple CP flow-through test using the LC_{50} estimates from the individual flow-through tests to calculate the a.t.u. This test yielded an LC_{50} value of 0.90 a.t.u., and a MTI value of 1.10 (Table 10). The 95 % confidence limits of the LC_{50} easily brackets 1 a.t.u. and we think the data fits the concentration addition model very nicely.

When we later repeated the 2,3,5,6 - TTCP static test, we got essentially the same LC_{50} as the initial test - 420 mg/l (95 % confidence limits, 316 - 558 mg/l). We can think of only two reasons why 2,3,5,6 - TTCP appears to be twice as toxic under static test conditions.

There is a relatively high loading density (5 juvenile fish per 3 liters of water) in the static jars. Dissolved oxygen levels may be somewhat lower (compared to flow-through

Table 09. Measured 96-hour LC_{50}s for 2,4,6- trichlorophenol, 2,3,5,6- tetrachlorophenol and pentachlorophenol to juvenile flagfish. [a]

CP compound	Concentration, mg/l		% trim
	LC_{50}	95 % confidence limits	
2,4,6 -TCP	2.207	1.995-2.441	0
2,3,5,6 - TTCP	1.164	1.040-1.303	0
PCP	0.218	0.197-0.243	0

[a] flow-through tests with acetone carrier at 79 mg/l.

Table 10. Acute toxicity of a triple CP mixture to juvenile flagfish. [a]

Test level	Concentration, acute toxic units [b] 2,4,6-TCP a.t.u.	2,3,5,6-TTCP a.t.u.	PCP a.t.u.	Total a.t.u.	Number exposed	Number dead
0	0	0	0	0	40	0
1	0.043	0.038	0.039	0.119	40	0
2	0.084	0.076	0.080	0.240	40	0
3	0.169	0.153	0.160	0.481	40	0
4	0.337	0.306	0.317	0.960	40	24
5	0.695	0.620	0.640	1.952	40	40
6	1.341	1.212	1.091	3.645	40	40

Trimmed Spearman - Karber Analysis

LC_{50}	95 % confidence limits	% trim	MTI value
0.899	0.807-1.002	0	1.10

a flow-through 96-hour test with acetone carrier at 79 mg/l.

b acute toxic units calculated from the concentration addition formula (Fig. 5).

conditions) and excess CO_2 could favour carbonic acid production, resulting in a depressed water pH. More of the TTCP compound would exist in the more toxic, undissociated form at a lower pH.

Or perhaps, a degradation product (either from metabolism or from physico-chemical breakdown) is being produced which is more toxic than the parent compound.

In any case, the toxic properties of 2,3,5,6 - TTCP under the two test conditions is an interesting phenonomen which we hope to persue at a later date. In the meantime, the next step as far as the acute testing is concerned, is to experimentally test the concentration addition model between the CB and CP classes of compounds. We think our acute data demonstrates that these chemicals are very close to being concentration additive within their own class. It will be interesting to see if the model is also applicable to a CB / CP mixture.

2. Chronic Testing.

In order to test any model for chronic joint toxicity, it is first necessary to bracket the MATC for a specific sublethal response in individual chronic tests performed on a mixture's components. Only when an MATC is bracketed can a chronic value be calculated for that particular parameter. The chronic values provide the database for converting the measured concentrations of the components in a mixture test to c.t.u., in the same way that LC_{50}s on separate components are used to convert the concentrations in a mixture to a.t.u.

A summary of the ELS data generated on individual CB

compounds is presented in Table 11. It is readily apparent from this table that we were not always successful in bracketing the MATC and hence, unable to calculate the critical number - the chronic value - for some of the sublethal responses measured in the ELS tests. One of the main problems encountered in establishing effective concentration gradients for individual CBs was the lack of reliable chronic data on these chemicals in the literature. More often than not, we had only our LC_{50} to use as a reference point.

The reported MATC values were, in most cases, boarderline in terms of the biological response being significant or non-significant. A shift of the exposure gradients up one concentration level would probably have resulted in successful bracketing of the no effect / effect intervals. Another problem with the ELS data on individual CB compounds is that there were no chronic tests performed on 1,2,4 - TCB. The TCB data presented in Table 11 is from previous work on dielectric fluids (Smith et al., 1979). This earlier study employed electrical grade TCB which is 66 % 1,2,4 - TCB and 33 % 1,2,3 - TCB.Therefore, the reported chronic values may not be an accurate estimate for pure 1,2,4 - TCB.

Despite these gaps in the chronic database we did proceed with a triple CB, ELS flagfish test selecting the concentration gradients for the compounds on the basis of the data in Table 11. At last year's conference, we presented the biological responses obtained in this test in the form of "interim" tables (Ozburn et al., 1982). We managed to bracket the MATC on 3 out of the 4 sublethal parameters measured, missing only the no effect /

Table 11. Summary of flagfish ELS data collected on individual CB compounds.

	Chronic value and / or MATC (ug/l)		
	1,4-DCB	TCB [a]	1,2,4,5-TTCB
Egg hatchability	MATC >835	chronic value 1432	MATC >238
10-day larval survival	chronic value 252 & 273	chronic value 1133	MATC >238
28-day fry survival	MATC >349	X [b]	chronic value 138
28-day fry growth	MATC >349	X	chronic value 85

[a] electrical grade TCB (data from Smith et al., 1979).

[b] no test performed.

effect interval for egg hatchability. However, time has not allowed us to repeat any of the individual tests, and without a complete set of chronic values, we are still unable to convert all of the measured concentrations to c.t.u.

Chronic testing on individual CP compounds is not finished at the present time (Table 12). Again, we already have the problem of an incomplete set of chronic values, even at this stage of the program.

We have also conducted a binary CB mixture test with 1,4 - DCB and 1,2,4 - TCB to evaluate flagfish 10-day larval survival. To demonstrate conversion of the chemical data from ug/l to c.t.u., the results of this test are presented in Table 13, utilizing chronic values of 252 ug/l for 1,4 - DCB and 1133 ug/l for 1,2,4 - TCB. It must be re-emphasized that the 1133 ug/l chronic value is based on electrical grade TCB and may not be an accurate estimate for pure 1,2,4 - TCB.

The CB binary test gave a chronic value of 1.17 c.t.u. (MATC, 1.01 - 1.36 c.t.u.). The corresponding MTI value is 0.77. We think this value is close enough to unity to suggest that concentration addition may in fact be an appropriate model for chronic joint toxicity of CBs. Repeat testing on individual compounds, to complete the chronic database, should be carried out to experimentally confirm the validity of the model.

3. Bioconcentration Testing

If, in fact, organic compounds like CBs bioconcentrate in fish with no interaction present, then we should be able to demonstrate that the parameters used to quantify bioconcentration

Table 12. Summary of flagfish ELS data collected on individual CP compounds.

	Chronic value and / or MATC (ug/l)		
	2,4,6-TCP	2,3,5,6-TTCP	PCP
Egg hatchability	test in progress	MATC >238	chronic value 136
10-day larval survival	test in progress	MATC >238	chronic value 75
28-day fry survival	test in progress	chronic value 136	chronic value 135
28-day fry growth	test in progress	MATC >1037	MATC >179

Table 13. Ten-day survival of flagfish larvae exposed to a binary chlorobenzene mixture.

Test level	Concentration 1,4 - DCB ug/l	1,4 - DCB c.t.u.	1,2,4 - TCB ug/l	1,2,4 - TCB c.t.u.	Total c.t.u.	Per cent survival
0	0	0	0	0	0	93
1	9.4	0.037	59.5	0.053	0.090	80
2	30.5	0.121	169.5	0.150	0.271	93
3	115.8	0.460	624.5	0.551	1.011	83
4	154.7	0.614	839.7	0.741	1.355	67 *
5	209.7	0.832	1118.0	0.987	1.819	47 **
6	316.0	1.254	1766.0	1.559	2.813	3 **

Lee-Desu Analysis

10-day survival - level 4(*) significant ($P < .05$)
- levels 5 and 6 (**) significant ($P < .01$)

Concentration, c.t.u.

Chronic value	MATC	MTI value
1.17	1.01 - 1.36	0.77

dynamics are approximately equal in magnitude when comparing individual tests to mixture tests. Flagfish bioconcentration data is presently available for individual tests with 1,4 - DCB and 1,2,4,5 - TTCB, as well as a mixture of 1,4 - DCB, 1,2,4 - TCB and 1,2,4,5 - TTCB.

The important bioconcentration parameters are: 1) the uptake rate constant, K_1; 2) the clearance rate constant, K_2; and, the bioconcentration factor, K_B. The experimental values obtained for these parameters in the individual CB tests are presented in Table 14. In order to demonstrate the structure - activity relationship of increasing bioconcentration potential with degree of chlorination, data on 1,2,4 - TCB (extracted from the triple CB mixture test) is also included. The values obtained for the same parameters in the triple CB mixture test are listed below in Table 15.

Both data sets nicely illustrate the structure - activity relationship. But if we compare the K_B values for DCB and TTCB from the individual tests to those obtained for the mixture, it is obvious that the bioconcentration factor for both CBs is considerably larger in the mixture test. This is contrary to what we would expect, assuming that no interaction is present. The K_B values should be approximately the same.

Because lipid is the target tissue or, "site of action" for bioconcentration of organic chemicals, we can take the same data sets from Tables 14 and 15 and express the values for the bioconcentration parameters on the basis of lipid content (Tables 16 and 17). Obviously, the K_B values have been transformed into much larger numbers. They can be compared much more easily,

Table 14.Bioconcentration of 1,4 - DCB, 1,2,4 - TCB [a] and 1,2,4,5 - TTCB in juvenile flagfish.

Bioconcentration parameter	mean ± s.d. 1,4-DCB	1,2,4-TCB	1,2,4,5-TTCB
Exposure [ ], ug/l	2.68±0.23	3.80±0.23	3.02±0.29
K_1, $days^{-1}$	291±26	1158±84	1630±246
K_2, $days^{-1}$	0.98±0.04	0.57±0.01	0.40±0.03
K_B	296±29	2026±154	4050±703

[a] data extracted from triple CB mixture test.

Table 15.Bioconcentration of a triple CB mixture in juvenile flagfish.

Bioconcentration parameter	mean ± s.d. 1,4-DCB	1,2,4-TCB	1,2,4,5,-TTCB
Exposure [ ], ug/l	3.28±0.27	3.80±0.23	3.87±0.27
K_1, $days^{-1}$	565±45	1158±84	1565±107
K_2, $days^{-1}$	1.32±0.04	0.57±0.01	0.22±0.01
K_B	429±37	2026±154	7048±606

Table 16.Bioconcentration of 1,4 - DCB, 1,2,4 - TCB [a] and 1,2,4,5 - TTCB in lipid of juvenile flagfish. [b]

Bioconcentration parameter	mean ± s.d. 1,4-DCB	1,2,4-TCB	1,2,4,5-TTCB
Exposure [ ], ug/l	2.68±0.23	3.80±0.23	3.02±0.29
K_1, $days^{-1}$	4230±439	10141±775	17100±3185
K_B	3590±498	17750±1481	50300±11427
log K_B	3.56±0.08	4.25±0.04	4.70±0.11

[a] data extracted from triple CB mixture test.

[b] K_B, K_1 lipid = 100/8.5 = 11.8 (K_B, K_1 body weight) and K_2 lipid = K_2 body weight.

Table 17.Bioconcentration of a triple CB mixture in lipid of juvenile flagfish. [a]

Bioconcentration parameter	mean ± s.d. 1,4-DCB	1,2,4-TCB	1,2,4,5-TTCB
Exposure [ ], ug/l	3.28±0.27	3.80±0.23	3.87±0.27
K_1, $days^{-1}$	4947±390	10141±775	13710±944
K_B	3760±318	17750±1481	61740±5232
log K_B	3.58	4.25±0.04	4.79

[a] K_B, K_1 lipid = 100/11.4 = 8.77(K_B,K_1 body weight) and K_2 lipid = K_2 body weight.

however, by coverting them to log numbers.

With the data now normalized on the basis of lipid weight, there does appear to be a relationship between individual CB bioconcentration and bioconcentration of CB mixtures. The mean log K_B for 1,4 - DCB and 1,2,4,5 - TTCB from the triple CB mixture can be readily bracketed by the standard deviations of the log K_B value for the same compounds in the individual CB tests. The standard deviations for the log K_Bs for these two components in the mixture test have been intentionally omitted to help clarify this relationship. Within the limits of experimental error, we can conclude that there is no interaction between components of a CB mixture; that is, the CBs bioconcentrate in fish independently of each other. Fig. 7 graphically illustrates both independent bioconcentration and the structure - activity relationship of the CB compounds.

If we also find that no interaction exists in the bioconcentration dynamics of CP compounds, we will attempt to develop a mathematical expression to quantify this relationship. The concentration addition model which has been developed for joint toxicity may very well be an appropriate approach towards quantifying joint bioconcentration.

Acknowledgments

Sam Spivak drafted the figures. This study is jointly supported by the Ontario Ministry of the Environment and by Environment Canada.

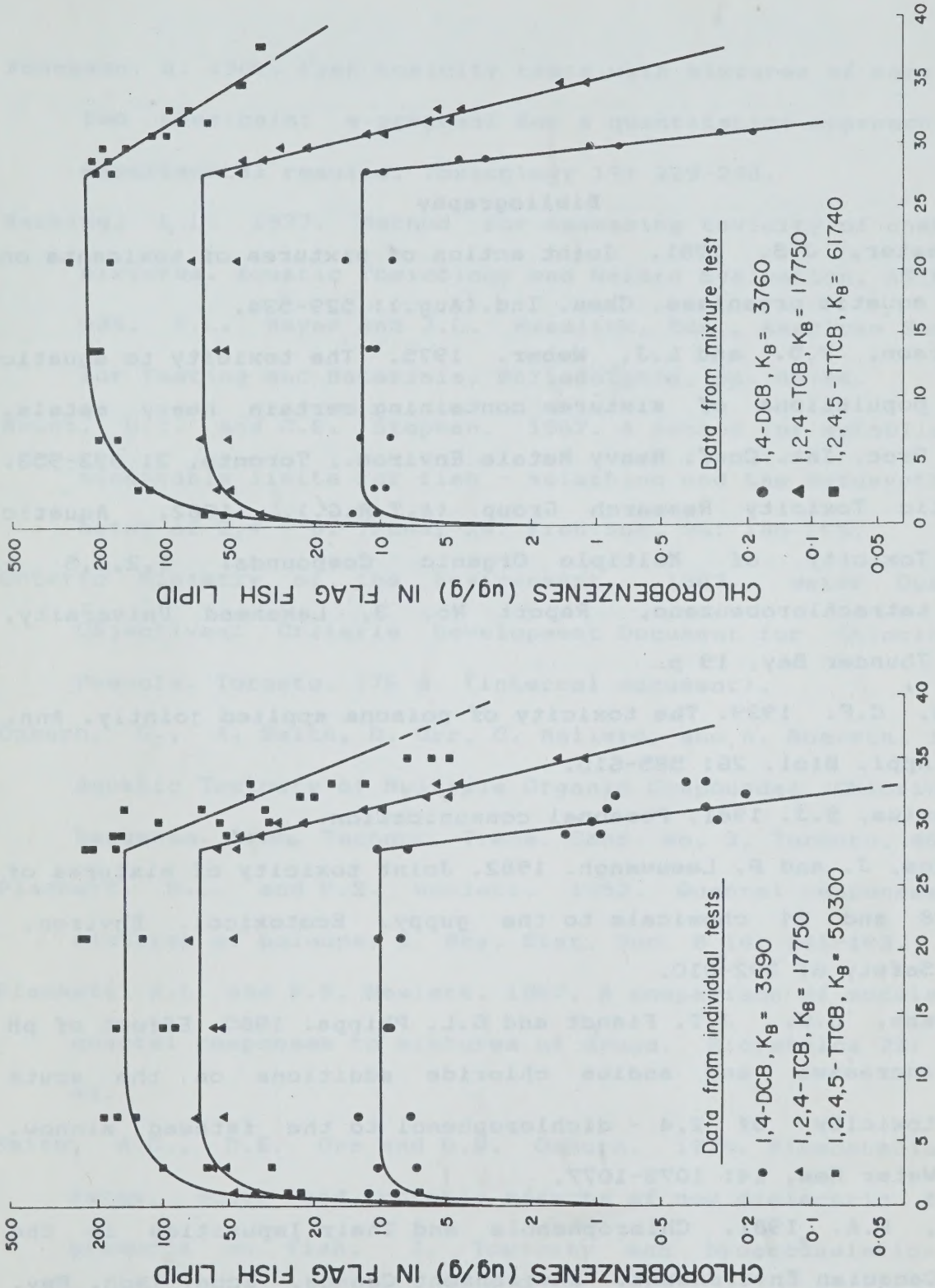


Fig. 07. Comparison between CB bioconcentration in individual compound tests and a triple mixture test.

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REMOVAL OF HAZARDOUS CONTAMINANTS (HCs) IN AN ONTARIO WATER POLLUTION CONTROL PLANT (WPCP)

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SUMMARY

The Ontario Ministry of the Environment (MOE) initiated a study to monitor HCs at an Ontario WPCP in 1982. The objective of the study was to provide an accurate estimate of the concentrations and annual loading of hazardous contaminants entering and being discharged from the WPCP, in order to determine the present sewage treatment process efficiency and factors influencing HCs removal. Parameters monitored included total PCBs, pesticides, PAHs, and trace metals. Conventional parameters were also monitored for reference purposes.

The study is still in progress, and therefore, a summary of the procedures, some analytical QA/QC results and preliminary treatment plant efficiency data will be presented for PAHs only.

ABSTRACT

Until very recently, relatively little was known about the identities and quantities of hazardous contaminants (HC's) entering wastewater treatment plants and little information was available on the factors which would influence their treatability and ultimate fate. Currently in Canada, the data base for HC's occurrence and removal at WPCP's is very limited. Therefore, in August 1982 a study was initiated to provide an accurate estimate of the annual loading of hazardous contaminants entering and being discharged from an Ontario WPCP, in order to determine the present sewage treatment process efficiency and factors influencing HC's removal. Monitoring included selected HC's such as PCBs, pesticides, PAHs, metals and conventional pollution parameters.

The study, which is still in progress, was divided into two phases. In Phase I, sampling was performed at the WPCP on two separate occassions in order to optimize site sampling techniques and analytical methods. Phase 2 involved collecting and analyzing 24 hour composite samples during three separate periods:

1. Winter (December 1982 and January 1983)
2. Spring (April and May 1983)
3. Summer (June to August 1983)

The selected parameters were determined in the raw combined influent and final effluent. The in-plant return was also monitored on certain sampling days. The WPCP operation was observed closely during the sampling periods and any operational upsets were recorded for correlation with the analytical results.

Chemical analyses and evaluation of the data are on-going. A progress report summarizing the study methodology and results available to date will be presented.

INTRODUCTION

Background and Relevance

In the modern industrial society, hazardous contaminants (HCs), both trace metals and organic compounds are discharged into public sewage systems. Until very recently, relatively little was known about the identities and quantities of contaminants entering wastewater treatment plants and little information was available on the factors which would influence their treatability and ultimate fate.

Recently published reports include four dealing with large scale field surveys and two based on studies of pilot-scale wastewater treatment facilities:

1. Survey of 40 Publicly Owned Treatment Works (POTW) by U.S. EPA Effluent Guidelines Division (EPA 1982a).
2. A 30 day study at a POTW by U.S. Effluent Guidelines Division (EPA 1982b).
3. Survey of 25 POTW by U.S. EPA Municipal Environmental Research Laboratory (MERL) (Cohen et al 1981).
4. The 5 plant study by the Chemical Manufacturers Association (CMA) and the U.S. EPA (CMA/EPA 1982).
5. U.S. EPA MERL pilot plant studies of semi-volatile organic compounds (Petrasek et al 1980 and 1981).
6. Pilot plant study of a group of volatile and semi-volatile organic compounds by van Rensburgh et al (1980) of the National Institute for Water Research in South Africa.

Results from the EPA surveys and pilot plant studies indicated that the wastewater treatment processes studied were stable over a wide range of operating conditions and were generally effective in removing toxic substances, as shown in Table 1.

However, the EPA 40 POTW study (EPA 1982a) showed that individual inorganics; e.g. As, Cd, Cu, Hg and Pb, and organics; e.g. polynuclear aromatic hydrocarbons (PAHs) and pesticides pass through a number of treatment plants in amounts and with frequency to be probable cause for concern.

TABLE 1. EXAMPLES OF TOXICS REMOVAL EFFICIENCY IN MUNICIPALS WPCPs

STUDY	REFERENCE	REMOVAL EFFICIENCY
40 POTW Survey	EPA (1982a)	For half of the plants studied: 70% for metals 82% for volatile organics 65% for base-neutral organics
25 POTW Survey	Cohen (1981)	>80% for many organics
Pilot Plant Study	Petrasek (1981)	>90% for the semi-volatiles studied

As influent concentrations of many conventional and priority pollutants increased, effluent concentrations also increased. This implies that the removal rates for the priority pollutants were relatively constant and that a fixed percentage of the loading of these pollutants was removed by secondary treatment.

In general, the higher the industrial contribution to a POTW, the higher the concentration of priority pollutants in the POTW influents. Heavy rainfall increased metallic priority pollutant mass loading at POTWs while the mass loading of both metallic and organic priority pollutants in POTW influents was higher on weekdays than on weekends.

Some pollutants not detected in POTW influents were regularly measured at high levels in the corresponding sludge streams; e.g. PAHs and phthalates which were concentrated to the greatest degree in sludges. In this regard, the survey data [EPA (1982a) and Cohen (1981)] support the findings of Petrasek (1981), who has suggested that sludges, particularly primary sludges are likely to act as a sink for these compounds because of a bioconcentration effect.

In the South African study (van Rensburg et al 1980), van Rensburg reported 90 percent effectiveness in the removal of toxic organics even under the pressure of shock loads of these chemicals, and he observed a severe build-up of some compounds in the recycled sludge. Both of these observations are in agreement with those reported by the U.S. EPA in their surveys and pilot-scale studies (EPA 1982a, Cohen 1981 and Petrasek 1981).

Trace organics monitoring studies at several Canadian sewage treatment plants are in progress (e.g. EPS 1980 and MOE 1980) and continuous flow fate studies of trace organics in a pilot plant at the Wastewater Technology

Centre (WTC) in Burlington, Ontario are on-going. However, currently in Canada the data base for HCs occurrence and removal at WPCPs is very limited (CANVIRO 1983). Therefore, in August 1982 a study was initiated to provide an accurate estimate of the annual loading of HCs entering and being discharged from an Ontario WPCP.

STUDY OBJECTIVES AND SCOPE

Analytical problems have been reported in many toxics studies conducted to date (e.g. EPA 1982a and CMA/EPA 1982), and therefore, the study was divided into two phases.

The specific objectives of Phase 1 were:

1. To identify the most significant HCs in the WPCP influent, effluent and sludge streams.
2. To establish a short list of HCs for the study's monitoring program.
3. To optimize site sampling techniques and analytical methods.

The monitoring program was conducted in Phase 2 and the objectives were:

1. To estimate the present sewage treatment process efficiency.
2. To determine the factors influencing HCs removal:

e.g. i) treatment process operating variables such as solids retention time (SRT),
ii) the levels of recycled HCs in the plant, and
iii) seasonal effects such as Spring run-off and dry weather periods.

Phase 1 - Contaminant Selection

The parameters selected for monitoring included PAHs, total PCBs, pesticides, heavy metals and conventional pollution parameters.

This study is still in progress, and therefore, the study procedures and preliminary results for the eight hazardous organic compounds shown in Table 2 will be presented.

TABLE 2. EIGHT HAZARDOUS CONTAMINANTS MONITORED AT THE WPCP

COMPOUND	CHEMICAL CLASSIFICATION
Acenaphthylene Benzo(a)pyrene Fluorene Fluoranthene Naphthalene Pyrene	Polynuclear Aromatic Hydrocarbons (PAHs)
Carbazole Dibenzofuran	Polynuclear Heterocyclic Compounds

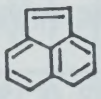
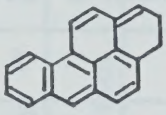
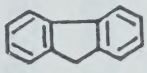
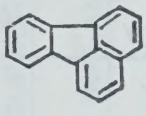
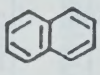
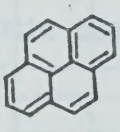
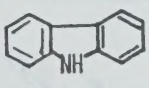
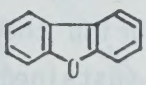
The chemical structures together with some physical data are provided in Table 3.

Environmental Significance

The environmental significance of these compounds can be summarized briefly as follows:

Although carbazole and dibenzofuran are not generally considered to be PAHs, they are structurally and chemically related to this group, as shown in Table 3. PAHs have been designated as priority pollutants by the U.S. EPA and in Canada they are included in the Water Pollution Control Directorate (WPCD) list of suspect/priority toxic chemicals. PAHs are probably the most widespread of all environmental contaminants (Jones and Leber 1979) and they have been identified in both Canadian and U.S. municipal treatment plants (Bridle 1982 and EPA 1982a). This group of compounds are non-polar and bio-resistant. They have an affinity for lipids, and therefore, they tend to bioaccumulate (Zedeck 1980). Some PAHs have been shown to be carcinogenic in laboratory studies (Thakker et al 1979), and yet although structurally similar, they have widely varying degrees of carcinogenicity (Daniel et al 1979). Benzo(a)pyrene is an important member of this class of compounds which has produced cytotoxic, mutagenic and carcinogenic effects (Jones et al 1979).

TABLE 3. STRUCTURAL AND PHYSICAL DATA FOR EIGHT HAZARDOUS CONTAMINANTS (EPA 1979)

COMPOUND NAME	COMPOUND STRUCTURE	MELTING POINT °C	BOILING POINT °C	VAPOUR PRESSURE AT 20°C torr	SOLUBILITY IN WATER AT 25°C mg/L	LOG OCTANOL/ WATER PARTITION COEFFICIENT
Acenaphthylene		92.3	265.8	10^{-3} - 10^{-2}	3.93	4.33
Benzo(a)pyrene		177	495	5 x 10^{-9}	0.0038	6.04
Fluorene		116-7	293-5	10^{-3} - 10^{-2}	1.98 1.69	4.18
Fluoranthene		111	375	10^{-6} - 10^{-4}	0.26	5.33
Naphthalene		80.5	218	0.0492	34.4 31.7	3.37
Pyrene		156	393	6.85 x 10^{-7}	0.14 0.132	5.32
Carbazole		247-8	355	N.A.	N.A.	N.A.
Dibenzofuran		86-7	287	N.A.	N.A.	N.A.

Notes: NA = Data Unavailable

STUDY PROCEDURES - Phase 1 and Phase 2

Sampling Methods

Twenty-four hour composite samples were collected on two occasions in Phase 1 and during three separate periods in Phase 2, as shown in Table 4.

TABLE 4. PHASE 1 AND PHASE 2 SAMPLING PROGRAM

PHASE	SAMPLING PERIOD	NUMBER OF SAMPLING DAYS
1	August - September 1982	2
2		
Winter	December 1982 - January 1983	6
Spring	April and May 1983	4
Summer	June - August 1983	4

The sampling locations in the WPCP are shown in Figure 1, and the following samples were collected:

1. Combined influent; i.e. raw sewage combined with the in-plant recycle stream.
2. Effluent.
3. Waste activated sludge (WAS).

The in-plant return stream was also sampled on an intermittent basis. The WPCP operation was observed closely during the sampling periods and the process data together with observations of sustained WPCP operational upsets were recorded for correlation with the analytical results for the parameters being measured.

This study was unique, in that the solid and liquid fractions of the samples were examined separately. Therefore, the samples were centrifuged to separate the two fractions ready for analysis. However, the suspended solids concentration of the effluent was extremely low, and in order to avoid centrifuging very large volumes of effluent, WAS solids were used instead.

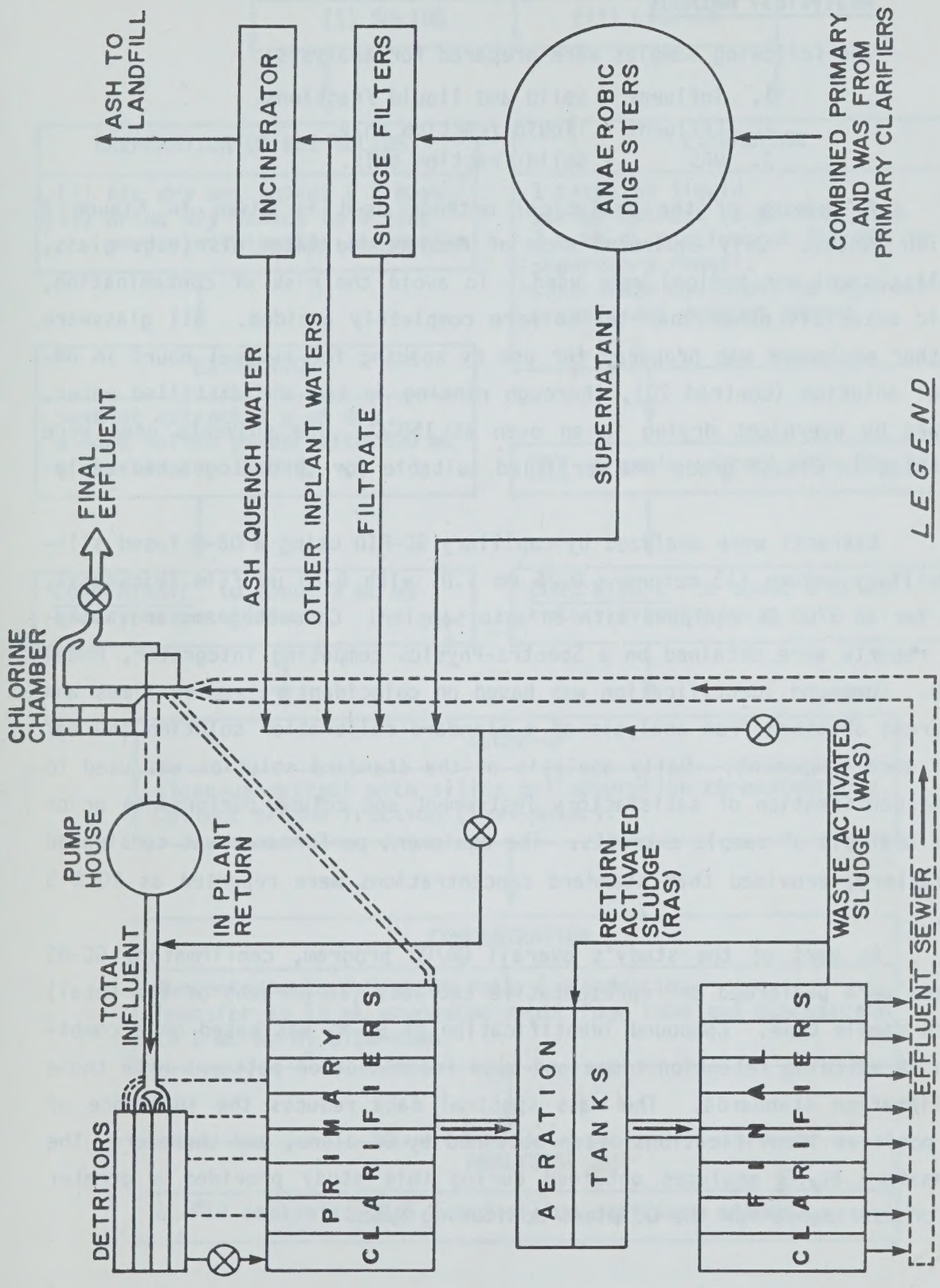


FIGURE 1 — WPCP PROCESS FLOW SCHEMATIC AND SAMPLING LOCATIONS

Analytical Methods

The following samples were prepared for analysis:

1. Influent - solid and liquid fractions.
2. Effluent - liquid fraction only.
3. WAS - solid fraction only.

A summary of the analytical methods used is given in Figure 2 (CANVIRO 1983b). Only equipment made of recommended materials (e.g. glass, stainless steel and teflon) were used. To avoid the risk of contamination, plastic materials other than teflon were completely avoided. All glassware and other equipment was prepared for use by soaking for several hours in detergent solution (Contrad 70), thorough rinsing in tap and distilled water, followed by overnight drying in an oven at 150°C. The solvents used were "Distilled in Glass" grade and certified suitable for chromatographic analyses.

Extracts were analyzed by capillary GC-FID using a DB-5 fused silica capillary column (15 meters x 0.25 mm I.D. with 0.25 um film thickness), and a Varian 3700 GC equipped with an auto sampler. Chromatograms and analytical reports were obtained on a Spectra-Physics computing integrator, Model SP4100. Compound identification was based on coincident retention times and peak areas obtained from analysis of a standard calibration solution (50 ng/uL) of each component. Daily analysis of the standard solution was used to provide confirmation of satisfactory instrument and column performance prior to the analysis of sample extracts. The equipment performance was considered satisfactory, provided that standard concentrations were reported as 50 ± 5 ng/uL.

As part of the study's overall QA/QC program, confirmatory GC-MS analyses were performed on representative extracts (18 percent of the total) of each sample type. Compound identification by GC-MS was based on a combination of matching retention times and mass fragmentation patterns with those of calibration standards. The mass spectral data reduces the incidence of false positive identifications often obtained by GC alone, and therefore, the confirmatory GC-MS analyses obtained during this study provided a greater level of confidence for the GC plant monitoring data.

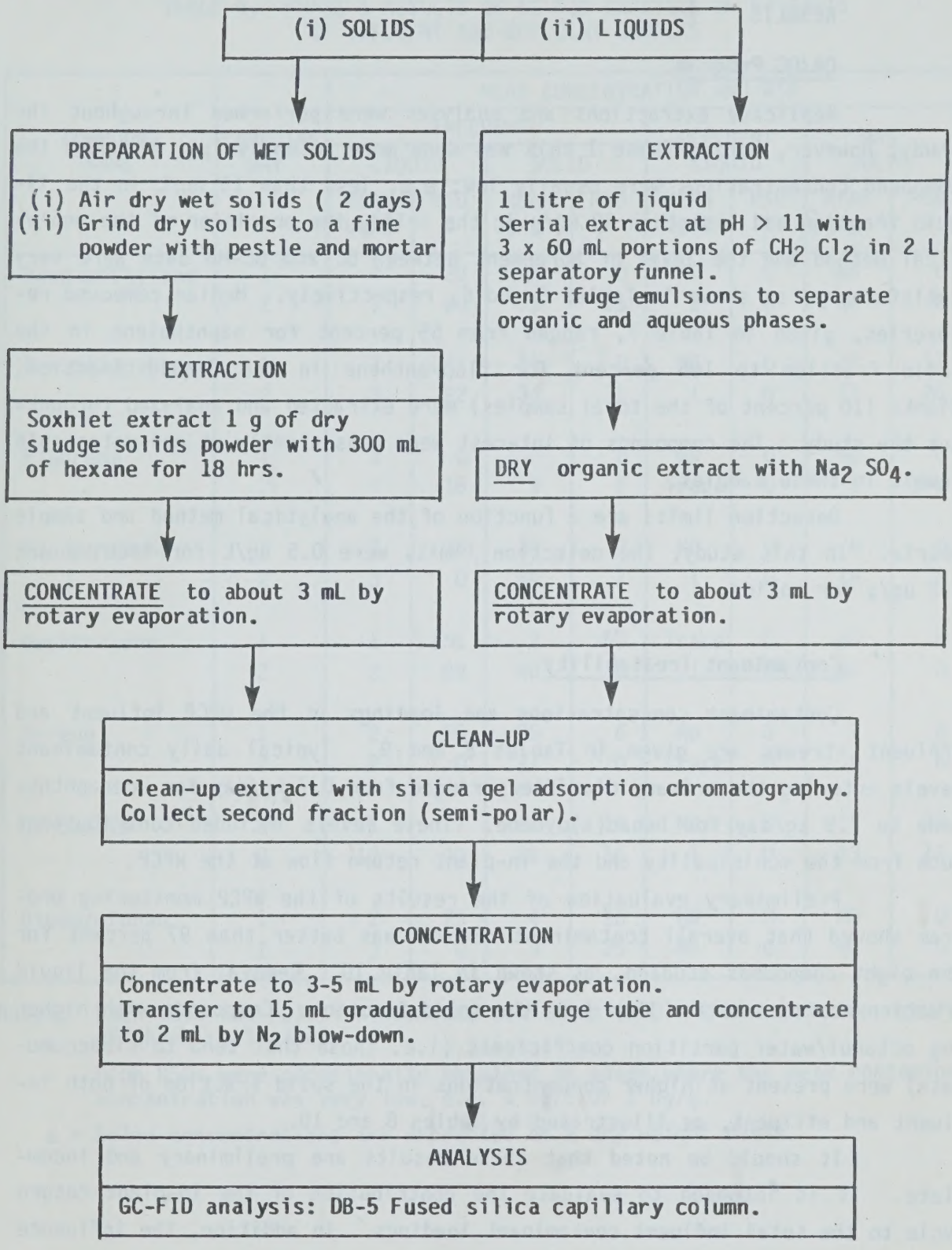


FIGURE 2: SUMMARY OF THE ANALYTICAL METHODS

RESULTS

QA/QC Program

Replicate extractions and analyses were performed throughout the study; however, during Phase 1 this was done more intensively. Although the compound concentrations were usually low; e.g. less than 15 ug/L in the liquid fraction and less than 50 ug/g in the solid, the precision of the analytical method and the level of agreement between GC and GC-MS data were very satisfactory, as shown in Tables 5 and 6, respectively. Median compound recoveries, given in Table 7, ranged from 65 percent for naphthalene in the solid fraction to 106 percent for fluoranthene in the liquid fraction. Blanks (10 percent of the total samples) were extracted and analyzed throughout the study. The compounds of interest were consistently at non-detectable levels in these samples.

Detection limits are a function of the analytical method and sample matrix. In this study, the detection limits were 0.5 ug/L for liquids and 1-2 ug/g for solids.

Contaminant Treatability

Contaminant concentrations and loadings in the WPCP influent and effluent streams are given in Tables 8 and 9. Typical daily contaminant levels entering the primary clarifiers ranged from 0.7 kg/day for acenaphthylene to 7.9 kg/day for benzo(a)pyrene. These levels included contributions both from the municipality and the in-plant return flow at the WPCP.

Preliminary evaluation of the results of the WPCP monitoring program showed that overall contaminant removal was better than 97 percent for the eight compounds studied, as shown in Table 10. Removal from the liquid fraction was much better than from the solid fraction. Compounds with higher log octanol/water partition coefficients (i.e. those that tend to bioaccumulate) were present at higher concentrations in the solid fraction of both influent and effluent, as illustrated by Tables 8 and 10.

It should be noted that these results are preliminary and incomplete. It is intended to evaluate the contribution of the in-plant return cycle to the total influent contaminant loadings. In addition, the influence of plant operating variables and seasonal conditions on treatment performance will also be determined.

TABLE 5. PHASE 1 RESULTS OF GC-FID ANALYSES OF EXTRACTS OF INFLUENT AND EFFLUENT SAMPLES

COMPOUND NAME	SAMPLING DAY	MEAN CONCENTRATION AND RSD							
		INFLUENT				EFFLUENT LIQUID		WAS SOLID	
		LIQUID		SOLID					
		MEAN ug/L	RSD %	MEAN ug/g [a]	RSD %	MEAN ug/L	RSD %	MEAN ug/g [a]	RSD %
Acenaphthylene	1	4	29	5	0	ND	0	ND	0
	2	5	20	5	25	1	0	ND	0
Benzo(a)pyrene	1	1	141	41	8	ND	0	27	17
	2	3	22	34	31	1	0	17	26
Fluorene	1	3	0	7	8	ND	0	ND	0
	2	4	16	9	7	Trace	0	1	172
Fluoranthene	1	3	35	37	2	ND	0	8	0
	2	3	0	44	7	1	0	14	8
Naphthalene	1	1	151	2	50	Trace	0	ND	0
	2	2	89	ND	0	1	0	ND	0
Pyrene	1	2	55	25	6	ND	0	7	8
	2	2	0	27	10	Trace	0	9	6
Carbazole	1	11	7	21	14	ND	0	11	22
	2	10	40	26	16	1	0	13	16
Dibenzofuran	1	2	29	4	16	ND	0	ND	0
	2	3	0	5	25	ND	0	ND	0

Notes: Each mean and relative standard deviation (RSD) is based on the results of three replicates.

High RSDs were occasionally obtained in cases where the mean contaminant concentration was very low; e.g. 1 ug/L or 1 ug/g.

a = Solid concentrations are expressed on a dry weight basis.

TABLE 6. COMPARISON OF RESULTS OBTAINED BY GC-FID AND GC-MS DURING PHASE 1 OF THE STUDY

COMPOUND NAME	SAMPLING DAY	CONCENTRATION							
		INFLUENT				EFFLUENT LIQUID ug/L		WAS SOLID ug/g	
		LIQUID ug/L		SOLID ug/g					
		GC	GC-MS	GC	GC-MS	GC	GC-MS	GC	GC-MS
Acenaphthylene	1	3	2	5	7	ND	ND	ND	Trace
	2	4	<10	4	4	1	ND	ND	ND
Benzo(a)pyrene	1	1	ND	38	23	ND	ND	26	16
	2	2	<10	36	5	1	ND	17	4
Fluorene	1	3	1	7	5	ND	Trace	ND	Trace
	2	4	<10	8	7	Trace	ND	2	ND
Fluoranthene	1	3	1	37	35	ND	Trace	8	7
	2	3	<10	43	47	1	ND	13	11
Naphthalene	1	ND	Trace	3	3	ND	Trace	ND	ND
	2	ND	ND	ND	1	1	<10	ND	1
Pyrene	1	2	Trace	24	19	ND	Trace	8	5
	2	2	<10	26	35	1	ND	9	13
Carbazole	1	12	NR	19	NR	ND	NR	12	NR
	2	14	NR	28	NR	1	NR	14	NR
Dibenzofuran	1	1	NR	4	NR	ND	NR	ND	NR
	2	3	NR	4	NR	ND	NR	ND	NR

Notes: GC analyses were performed by CANVIRO CONSULTANTS LTD.

GC analyses were performed on a Finnigan 1020 GC-MS at the Wastewater Technology Centre in Burlington, Ontario.

Solid concentrations are expressed on a dry weight basis.

NR = No result; compound not measured.

ND = Not detected.

Trace = Compound was detectable, but the concentration was too low to measure.

TABLE 7. SUMMARY OF RECOVERIES OF COMPOUNDS FOR SAMPLE SOLID AND LIQUID FRACTIONS

COMPOUND	SAMPLE FRACTION	PERCENT RECOVERIES		
		MEDIAN	20% [a]	80% [a]
Acenaphthylene	solid	82	75	88
	liquid	87	81	98
Benzo(a)pyrene	solid	78	58	83
	liquid	102	96	120
Fluorene	solid	89	78	92
	liquid	95	90	102
Fluoranthene	solid	88	80	96
	liquid	106	97	112
Naphthalene	solid	65	57	75
	liquid	80	68	86
Pyrene	solid	67	59	71
	liquid	102	96	105
Carbazole	solid	82	68	98
	liquid	102	86	107
Dibenzofuran	solid	82	78	90
	liquid	97	94	111

Notes:

Recoveries were determined at two levels:

i) 50 ug/g and 100 ug/g for solid samples.
ii) 50 ug/L and 100 ug/L for liquid samples.

Recovery data is based on 14 replicates for each sample fraction.

a = Recoveries were calculated at the 20 and 80 percentiles.

TABLE 8. MEDIAN CONCENTRATIONS OF COMPOUNDS IN INFLUENT AND EFFLUENT SAMPLES

COMPOUND	MEDIAN CONCENTRATION					
	INFLUENT			EFFLUENT		
	SOLID FRACTION ug/g	LIQUID FRACTION ug/L	% OF TOTAL DUE TO SOLIDS CONTRIBUTION	SOLID FRACTION ug/g	LIQUID FRACTION ug/L	% OF TOTAL DUE TO SOLIDS CONTRIBUTION
Acenaphthylene	6	2	32	Trace	ND	100
Benzo(a)pyrene	99	5	80	22	ND	100
Fluorene	8	2	50	Trace	ND	100
Fluoranthene	28	1	85	16	ND	100
Naphthalene	5	1	28	1	Trace	NC
Pyrene	27	1	85	21	ND	100
Carbazole	38	10	43	22	ND	100
Dibenzofuran	6	2	47	Trace	ND	100

Notes:

ND = Not detected

NC = Not calculated. Naphthalene in the liquid fraction was detectable, but the concentration was too low to measure.

TABLE 9. CONCENTRATIONS AND LOADINGS OF COMPOUNDS IN TOTAL INFLUENT AND EFFLUENT

COMPOUND	PARAMETER	TOTAL INFLUENT		TOTAL EFFLUENT	
		CONCENTRATION ug/L	LOADING kg/day	CONCENTRATION ug/L	LOADING kg/day
Acenaphthylene	Median	3	0.7	Trace	Trace
	No. of Detections	16/16		11/16	
Benzo(a)pyrene	Median	27	7.9	Trace	Trace
	No. of Detections	16/16		15/16	
Fluorene	Median	4	1.0	Trace	Trace
	No. of Detections	16/16		10/16	
Fluoranthene	Median	8	2.4	Trace	Trace
	No. of Detections	16/16		16/16	
Naphthalene	Median	3	1.1	Trace	Trace
	No. of Detections	16/16		12/16	
Pyrene	Median	7	2.1	Trace	Trace
	No. of Detections	16/16		16/16	
Carbazole	Median	19	6.2	Trace	Trace
	No. of Detections	16/16		15/16	
Dibenzofuran	Median	3	0.8	Trace	Trace
	No. of Detections	16/16		8/16	

Notes:
Trace Loading = Less than 0.1 kg/day (average plant flow = 300.000 m^3/day)
No. of Detections = Number of days detected during 16 day monitoring program.

TABLE 10. PERCENT REMOVAL OF COMPOUNDS BY WPCP TREATMENT

COMPOUND	MEDIAN PERCENT REMOVAL		
	SOLID FRACTION	LIQUID FRACTION	TOTAL
Acenaphthylene	97.5	100	99.9
Benzo(a)pyrene	71.1	100	98.7
Fluorene	98.6	100	99.9
Fluoranthene	49.1	100	97.8
Naphthalene	100	93.3	99.7
Pyrene	39.2	100	97.5
Carbazole	43.5	100	98.4
Dibenzofuran	99.9	100	100

CONCLUSION

Treatment Plant Performance

Trends in the treatment plant performance shown by these preliminary results are in good agreement with those obtained in the recent EPA 40 POTW study (EPA 1982a). Whilst PAHs were adsorbed onto the solids fraction, and therefore were largely removed during primary treatment, low levels of these compounds were measured in the WAS solids. Thus, whilst overall removability of these compounds by the WPCP was very good (>97 percent), detectable traces were discharged in the effluent (<.04 ug/L by estimation). On one isolated occasion during the sixteen days of the monitoring program for reasons yet to be identified, much higher levels than normal of these compounds (e.g. 413 ug/L compared to <25 ug/L of fluoranthene) were measured in the influent. On this occasion, slightly increased levels were observed in the effluent (e.g. 3 ug/L compared <1 ug/L of fluoranthene). This is in keeping with the EPA results (EPA 1982a), which suggest that the removal rate for most contaminants appeared to be fairly constant, a fixed percentage of the influent loading being removed during the treatment process.

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